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Genetic Elements of Plant Viruses as Tools for Genetic Engineering

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INTRODUCTION

Viruses exploit their host cells as a source of energy, preformed constituents, and biosynthetic machinery. Efficient replication and expression of a virus genome are achieved through the competition for these resources with cellular genes. For systemic infection, virus encapsidation and spread of progeny must follow before the virus is neutralized by the defense systems of the host. To fulfill these tasks, viruses must rely upon the limited coding capacities of their genomes. In particular, genome sizes in viruses of higher plants, the subject of this review, are mostly within the range of 1 to 10 kb, regardless of the type of virion nucleic acid or expression strategy. The genetic elements that govern expression of virus genetic systems are therefore by necessity highly efficient, often small, and

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multifunctional. These properties make plant viruses useful models for the study of minimal forms of expression of genetic information in plant cells, and they also make them a good source of sequences of value for plant genetic engineering.

The scope of this review is to summarize the available data, as of May 1995, on the sequences derived from plant DNA and RNA viruses that have been shown to control expression of virus genetic information or of heterologous genes in artificial fusions. The implications of these studies for the controlled expression of transgenes in plants will also be considered. Moreover, we will discuss the use of engineered plant virus genomes as self-propagating vectors for the expression of heterologous genes in vivo. We will also address the question of stability of plant virus-based replicons.

We chose to focus on the general applicability of plant virus sequences in plant molecular biology. Therefore, examples of virus-derived plant resistance, in which transgenic plants that express portions of virus genomes appear to be protected against virus infection, will not be covered here. Two types of genetic elements applicable in genetic engineering that will not be discussed are genes from viruses of lower plants, in particular from algal DNA viruses that contain strong promoters and encode a variety of DNA modification and restriction enzymes (239), and ribozymes encoded by some viroids and satellites of plant RNA viruses (see, e.g., references 49, 284, and 307). On the other hand, many plant and animal viruses share profound similarities in sequences of certain essential genes and also in the modes of genome expression (185, 285), and we will use data on better-studied animal virus systems when they shed light on the effects observed in the related plant viruses.

Since the appearance of a book chapter (318) with a very similar scope, new data have been obtained, so we believe that the present review is timely. There are excellent recent reviews that cover in depth some of the relevant areas, such as transcription in caulimoviruses (295), the recoding of RNA in viruses (11, 123, 147, 202), virus-encoded proteolytic activities (88), and biotechnological aspects of geminiviruses (28, 29, 230, 336). Although the same issues are discussed below in some detail, the reader is encouraged to use these sources for more specific information and for many additional references.

CONTROL ELEMENTS ENCODED BY PLANT VIRUS GENOMES

Control of Plant Virus Nucleic Acid Synthesis

Plant viruses and their features discussed in this review are listed in Table 1. Whereas the majority of known plant viruses are RNA viruses that do not possess a DNA intermediate in their life cycle, it is the smaller division of plant viruses with virion DNA that is better known to genetic engineers, because cauliflower mosaic virus (CaMV), the type and best-studied member of the caulimovirus group, served as the source of one of the first-characterized plant promoters and polyadenylation signals (20–23, 177, 193, 194, 244, 297, 304a).

Caulimoviruses are plant pararetroviruses that replicate their 8-kbp DNA genome via the reverse transcription of the genome-length, terminally redundant RNA (for reviews, see references 57, 285, and 295). One of the two CaMV promoters, the 35S promoter, and its engineered derivatives are very efficiently transcribed by cellular enzymes and have been extensively used to drive a high-level, near-constitutive expression of many heterologous genes in transient-expression assays and in several species of transgenic plants (see, e.g., references 1, 14, 20–23, 297, and 371). Dissection of the 35S promoter sequences has allowed delineation of several elements that direct tissue-specific gene expression through interaction with different host-encoded transcriptional factors (20–23, 176, 193, 194). Another CaMV promoter, the 19S promoter, is much weaker than the 35S promoter, and the activity of the former in CaMV is thought to be regulated at a distance by the latter (92). Promoters from the badnaviruses, the second group of plant pararetroviruses, are also able to govern strong expression of the heterologous genes (25, 217, 218). The *cis* element that controls termination of synthesis and polyadenylation of both caulimovirus transcripts has also been studied in some detail (286, 295, 296).

Geminiviruses, another group of plant viruses with virion DNA, are the source of promoter sequences that appear to be strong and constitutive in some tissues of their host plants, whereas in other tissues, virus-encoded transcriptional *trans*-activators are required for their full activity (27, 142, 289, 327). These elements provide diverse opportunities to engineer transgenes with controlled levels, or tissue specificity, of expression. Possibilities of such control at the level of DNA-dependent RNA synthesis will be considered first in this section. Geminiviruses replicate their DNA genome in the host nucleus in a manner unusual for the nuclear setting, namely, by the rolling-circle mechanism (161, 186, 323, 325). We will consider virus proteins and *cis* sequences known to be involved in this process.

The majority of plant viruses do not rely on the nuclear transcriptional machinery in their life cycle. Instead, they replicate their RNA genomes by RNA-dependent RNA synthesis, mediated by replicative complexes formed by both virus-encoded and host-encoded proteins in the cytoplasm. Structures at the termini of virus genomic RNAs are particularly important for full-length genome synthesis (RNA replication). Sequences located internally on the virus genomic RNAs mediate synthesis of subgenomic RNAs (a process referred to as RNA-dependent RNA transcription) and in some cases affect full-length genome replication as well. Characterization of these *cis*-acting sequences permits construction of virus-based mini-replicons that can be propagated in the presence of enzymatic activities provided in *trans* either by a helper virus or by transgenic plants. The RNA-directed RNA synthesis will be discussed with the focus on the mechanisms involved and then again in the section dealing with self-replicating plant virus vectors.

DNA-directed RNA synthesis. (i) Plant pararetroviruses. Both known groups of plant pararetroviruses, spherical caulimoviruses and bacilliform badnaviruses, possess relaxed circular double-stranded (ds) DNA in the virion. One (the minus) strand of virion DNA contains a single interruption, whereas the other (plus) strand can be interrupted at one, two, or three sites (57). These interruptions are site specific and are thought to arise as a result of the reverse transcription mechanism (see references 57 and 285 for details). The breaks in DNA are repaired upon entry of the virus into the host nucleus, as shown for CaMV (247). As a result, a covalently closed virus minichromosome that can be transcribed by a host RNA polymerase II-like enzyme is formed (247). This step is apparently the only multiplicative stage in plant pararetrovirus reproduction, so it is not unexpected that transcription levels from plant pararetrovirus promoters are very high.

Upon caulimovirus infection, two major transcripts are detected. One transcript is the more-than-genome-length RNA, containing a terminal redundancy of ca. 180 nucleotides (nt). The other transcript is the subgenomic RNA for translation of virus gene VI (243). The two transcripts are 3′ coterminal. Neither a counterpart of gene VI nor the subgenomic promoter has been found in badnaviruses thus far; accordingly,

TABLE 1. Names, taxonomic positions, and features of the plant viruses discussed in this review

Virus	Virus group[a]	Relevant genetic elements or features of the lifestyle
African cassava mosaic virus	Geminivirus (III)[b]	Activation of promoter by the AC2 protein
Alfalfa mosaic virus (AlMV)	Alfamovirus	Binding of the capsid protein to the positive-strand RNAs is essential for initiation of virus replication 5′- and 3′-UTRs of the subgenomic RNA 4 enhance translation
Barley stripe mosaic virus	Hordeivirus	Vector (autonomous; replacement; protoplasts; reporter)
Barley yellow dwarf virus (strain PAV) (BYDV-PAV)	Alloluteovirus[b]	Cap-independent initiation of translation (5′ and 3′ signals) Leaky ribosome scanning Translational readthrough (−1) translational frameshift (5′ and 3′ signals)
Barley yellow mosaic virus	Bymovirus	VPg at the 5′ end of the genomic RNA NLS in the capsid protein
Beet curly top virus	Geminivirus (II)	Evidence for the rolling-circle mechanism of replication
Beet necrotic yellow vein virus	Benevirus	5′ and 3′ sequences on the genomic RNA 3 essential for its replication RNA 3-encoded cryptic ORF X can induce cell death
Beet yellows virus	Closterovirus	+1 translational frameshift in the polymerase gene
Broad bean mottle virus	Bromovirus[b]	Recombinational synthesis of the DI virus replicons is strongly reduced in some hosts
Brome mosaic virus (BMV)	Bromovirus[b]	Multisubunit structure of the RNA replication complex 5′- and 3′-UTRs on the genomic RNA components, including 3′ tRNA-like structure, required for recognition by the replication enzyme Overlapping internal sequences on RNA 3 required for its own synthesis and for synthesis of the subgenomic RNA 4 Vector (autonomous; insertion and replacement; protoplasts; reporter) Replication of the RNA 3 and its engineered derivatives in transgenic plants and in yeasts that express virus replicative proteins Chimeric RNA 3 that contains capsid protein gene of the cowpea strain of TMV and its cognate origin of assembly sequence is encapsidated into TMV-like rodlets RNA recombination by the copy choice mechanism is not strongly sequence dependent but requires base pairing between the two recombining molecules and the template
Cauliflower mosaic virus (CaMV)	Caulimovirus[b]	35S promoter, the upstream enhancer, and the derivatives of those; organ-specific activity of the 35S derivatives; 19S promoter; interactions of the two promoters dependent on their relative position 35S polyadenylation signal Translation of the downstream genes from the polycistronic RNAs is mediated by the product of ORF VI Vector (autonomous; replacement; whole plants; reporters, heavy-metal tolerance, interferon); replication of two disabled genomes by mutual complementation Virus genome as a genetic marker in agroinfection or for intrachromosomal DNA recombination
Chloris striate mosaic virus	Geminivirus (I)[b]	Enhancement of transcription from the weak capsid protein promoter by the capsid protein and by the C1-C2 replication protein
Citrus tristeza virus	Closterovirus	+1 translational frameshift in the polymerase gene
Coconut foliar decay virus		Phloem-specific promoter
Commelina yellow mottle virus (CoYMV)	Badnavirus	Strong tissue-specific promoter; transcriptional enhancer
Cowpea chlorotic mottle virus	Bromovirus[b]	Intercistronic sequences on RNA 3 required for RNA synthesis—differences with BMV Recombination of the deleted virus genome with a missing virus gene expressed by a transgenic plant—biosafety concerns
Cowpea mosaic virus (CPMV)	Comovirus	5′-terminal VPg Controversy concerning the mechanism of cap-independent initiation of translation Genome expression by proteolytic processing of the polyproteins Terminal sequences on the M RNA component required for its replication *trans* replication of M RNA and *cis*-preferential replication of B RNA component Antigen display on the surface of virions
Cucumber mosaic virus (CMV)	Cucumovirus	RNA replicase complex that enables a complete round of virus RNA replication in vitro Terminal and internal sequences on the RNA 3 required for recognition by polymerase complex

Continued on following page

TABLE 1—*Continued*

Virus	Virus group[a]	Relevant genetic elements or features of the lifestyle
Cucumber necrosis virus	Tombusvirus	Increase in DI RNA synthesis by knockout of a nonessential p20 protein
Cymbidium ringspot virus	Tombusvirus	Vector (nonautonomous, DI RNA based; insertion; whole plant; capsid proteins of heterologous viruses)
Figwort mosaic virus (FMV)	Caulimovirus[b]	Strong promoter
Maize chlorotic mottle virus	Machlovirus	−1 translational frameshift
Maize streak virus (MSV)	Geminivirus (I)[b]	Head-to-head promoters enhanced bidirectionally by a discrete sequence element Vector (autonomous; insertion; whole plant; reporter gene, expressed only locally)
Odontoglossum ringspot virus	Tobamovirus[b]	Subgenomic promoter used in the TMV vectors to reduce loss of an insert
Pea enation mosaic virus	Enamovirus	VPg; −1 translational frameshift
Peanut chlorotic streak virus	Caulimovirus[b]	Strong genomic promoter Potentially higher insertion capacity than in CaMV vector
Plum pox virus	Potyvirus[b]	Shown helicase activity of the replication protein CI
Potato virus X (PVX)	Potexvirus[b]	5′-UTR on the genomic RNA acts as a translational enhancer; motifs required for this activity Vector (autonomous; insertion; whole plants; reporter)
Potato virus Y (PVY)	Potyvirus[b]	5′-UTR promotes internal initiation of translation
Rice tungro bacilliform virus	Badnavirus	Strong tissue-specific promoter with the upstream and downstream transcriptional enhancers
Satellite tobacco necrosis virus		5′- and 3′-UTRs enhance translation synergistically
Soybean chlorotic mottle virus	Caulimovirus[b]	Strong promoters
Tobacco etch virus (TEV)	Potyvirus[b]	5′-UTR is a cap-independent translational enhancer Discrete NLSs in the NIa protein NIa protein is a serine-type protease with high specificity; NIa-based polyprotein cleavage cassettes Vector (autonomous; insertion; whole plants; reporter) Replication of a disabled virus in transgenic plants expressing NIb RNA polymerase
Tobacco mosaic virus (TMV)	Tobamovirus[b]	3′-terminal tRNA-like structure is required for recognition of RNA polymerase Artificially constructed defective replicons can be propagated in the presence of wild-type virus Subgenomic promoters of the MP gene and of the capsid protein gene control different levels and different temporal patterns of expression of their corresponding subgenomic RNAs 5′-UTR is a translational enhancer tRNA-like structure and a 3′-terminal pseudoknot are required for maximal activity of the 5′ translational enhancer The rate of translational readthrough of the 126-kDa replication protein is determined primarily by two codons downstream of the leaky termination codon 30-kDa MP interacts with the plasma membrane or cell wall and increases plasmodesmatal size exclusion limit Certain modifications in the capsid protein elicit necrotic response in *Nicotiana* cells that contains gene N—may be useful as a model for programmed cell death in plants Self-assembly of the capsid protein and any RNA that contains virus origin of assembly—a system for selective rescue and storage of the tagged transcripts Vector (autonomous; insertion; whole plants; numerous foreign genes); epitope presentation Use of virus vector with the inserted selectively neutral gene for the estimation of the mutation rate in plant virus RNA replicons
Tomato bushy stunt virus	Tombusvirus	Vector (autonomous; replacement; whole plant, accumulates only locally; reporter)
Tomato golden mosaic virus (TGMV)	Geminivirus (III)[b]	AL2 gene product is a transcriptional *trans*-activator of the AR1 and BR1 promoters AL1 gene product represses activity of its own promoter Vector (autonomous; replacement; whole plants; reporter)
Turnip crinkle virus	Carmovirus	Structures on RNA that are required for sequence-specific RNA recombination Discrete RNA sequences required for virion assembly
Turnip yellow mosaic virus (TYMV)	Tymovirus	The first-described tRNA-like structure at the 3′ end of virus RNA; mutations in the anticodon loop and other regions of this structure strongly reduce virus replication *cis*-preferential replication Leaky initiation enables translation of the two extensively overlapping ORFs
Wheat dwarf virus (WDV)	Geminivirus (I)[b]	Characterization of *cis* sequences required for initiation of DNA synthesis Vector (autonomous; replacement; protoplasts; reporter); shuttle vector capable of replicating both in plant cells and in bacteria

[a] Names of the virus groups are those in reference 105 with changes proposed in reference 185.
[b] The genome of at least one member of this group is represented schematically in one of the figures.

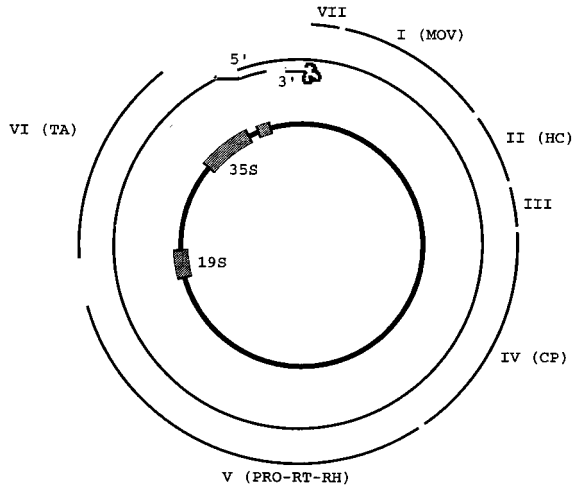

FIG. 1. Organization of the genome of CaMV (caulimovirus group). Virion ds DNA of 8 kbp is represented by the innermost circle. The genome-length, terminally redundant 35S transcript is shown by the middle interrupted circle. The cloverleaf indicates the cell-encoded methionyl-tRNA that primes minus-strand DNA synthesis at a specific position close to the 5′ terminus of the 35S RNA. Black lines indicate the positions of virus ORFs. Shaded boxes indicate the positions of two virus promoters (35S and 19S) and polyadenylation signal. MOV, cell-to-cell movement protein; HC, helper component required for aphid transmissibility of virus particles; CP, major capsid protein; PRO-RT-RH, polyprotein containing aspartic protease, reverse transcriptase, and RNase H domains; TA, *trans*-activator protein.

the terminally redundant genomic transcript is the principal badnavirus-specific RNA (57). Additional minor transcripts and sequences with some degree of promoter activity have been reported for several caulimoviruses (145, 258) but have not been characterized in detail.

In CaMV, the larger transcript and the RNA for open reading frame (ORF) VI expression are called the 35S RNA and 19S RNA, respectively, after their experimentally determined sedimentation constants. We will refer to analogous promoters in other caulimoviruses as the genomic and subgenomic promoters, although other designations also have been proposed (277, 299).

(ii) 35S promoter of CaMV. The well-studied 35S promoter of CaMV was initially characterized as a ca. 350-bp DNA fragment spanning the very 3′ end of CaMV gene VI and part of the large intergenic region (244) (Fig. 1). When fused to various reporter genes, this segment was shown to confer strong expression in tobacco calluses regenerated from the transformed cells. Sequences upstream of this stretch or downstream of the +8 position (where +1 is the transcription start) did not affect properties of the promoter (244). Extensive analysis of the 35S promoter has shown that it can be viewed as a set of modules with distinct biological properties.

The sequence from −46 to +8 contains the conventional proximal element of eukaryotic promoters (the TATA box) and is sufficient for the accurate initiation of transcription, albeit at low level compared with the full-length promoter (244). The sequence from −90 to +8 (domain A [20]) enables strong expression of a reporter gene in the roots of transgenic tobacco and much weaker expression in the aerial parts of a plant (20, 21). The area between −90 and −46 apparently contains determinants for activation of the TATA box and for root-specific expression. Two CCAAT box-like sequences and two TGACG motifs are found in this segment; only the TGACG motifs are essential for both activities (193, 194). When the element from −82 to −62 of the 35S promoter is fused to the promoter of the small subunit of the ribulose 1,5-diphosphate carboxylase gene that was preferentially expressed in green tissues, the expression from the chimeric promoter was slight in leaves but strong in roots (193).

The stretch of sequence from −343 to −90 (domain B), when combined with domain A, restored strong constitutive expression of a reporter gene in most aerial parts of transgenic tobacco, excluding petals and some parts of the embryo (20). Interestingly, expression in roots was observed even if domain B was combined with the −46 to +8 portion of domain A (20). Further analysis revealed that the subdomains can be defined within domain B that will confer distinct patterns of tissue-specific and developmentally regulated expression when individually fused either to domain A or to its −46 to +8 derivative (20–22). The effects of some subdomains are subtle; in contrast, the B3 subdomain (−208 to −155) combined with domain A governs constitutive expression in tobacco. Some of the subdomains act independently of each other, so that the combination of two subdomains produces an expression pattern that is the sum of the two individual patterns; other pairs of subdomains act synergistically rather than additively (20, 98). Although the borders of the subdomains were arbitrarily chosen for the convenience of engineering, these data illustrate the broad possibilities of developing virus-derived promoters of chosen specificity.

Interestingly, in transgenic petunia, the expression patterns of many 35S promoter derivatives deviated from what had been observed in tobacco. For example, in petunia, several subdomains of B combined with domain A conferred strong expression of reporter genes in many tissues of the flower petals, a result never obtained with tobacco (20). This observation indicates that different sets of host factors interact with *cis* elements of the promoters even in the related hosts. Transcription factors which bind specifically to discrete portions of the CaMV 35S promoter have been characterized (176, 193, 194), but the molecular basis for the differential interaction of promoter with various host proteins is still unknown. Thus, the 35S promoter of CaMV contains modules useful for engineering differentially expressed gene fusions, but the optimal combination of subdomains must be reestablished for each plant species. Likewise, the full-length 35S promoter is presumed to be constitutive, but this might not be altogether true for every plant or tissue.

Further modifications of the 35S promoter of CaMV have created new useful promoters. A DNA sequence element of about 170 bp has been isolated from the promoter of the α′ subunit of the soybean β-conglycinin gene; this segment was inserted upstream of the −90 segment of the 35S promoter; the resulting chimeric promoter was able to enhance the otherwise low expression of a reporter gene in seeds of transgenic tobacco by 25- to 40-fold (53). The same element also possessed some activity (two- to fourfold enhancement) when positioned downstream of the reporter gene (53). Thus, the CaMV promoter or, at least, its A domain is responsive to tissue-specific, position-independent transcriptional enhancement.

A derivative of the 35S promoter has been recently engineered to carry three copies of a bacterial sequence, the 19-bp palindromic *tet* operator from the Tn*10* transposon (122). Tobacco plants were transformed with the β-glucuronidase (GUS) reporter gene under control of this chimeric promoter and also with the bacterial *tet* repressor gene. In such double transgenic plants, the 35S promoter is repressed; in the presence of the inducer tetracycline, the promoter is derepressed and an impressive 500-fold increase in reporter activity is observed (122). In a similar manner, 35S promoter activity in

tobacco protoplasts was enhanced by inserting an animal virus-derived DNA element into a plant expression cassette after providing a transcriptional activator in *trans* (364).

In another work, the −343 to −90 activator sequence was tandemly repeated and inserted upstream of the full-length CaMV 35S promoter (177). The resulting promoter directed 10-fold-higher expression of a reporter gene in transgenic tobacco plants than the native 35S promoter. Interestingly, an effect of this double enhancer was also observed on the adjacent genes of the nopaline T-DNA that was used for plant transformation (177). Versions of the CaMV 35S promoter with the double enhancer are now widely used whenever strong constitutive expression of a transgene is desirable.

Notably, neither tobacco nor petunia is a host for CaMV, yet the 35S promoter of CaMV is among the strongest known promoters in these plants, as well as in many other nonhost dicot and monocot plants (14, 371). Moderate activity of the 35S promoter has also been observed in *Escherichia coli* (9), in *Schizosaccharomyces pombe* (126), and in *Saccharomyces cerevisiae* (290). In the latter host, the 35S promoter activity is increased upon nutritional depletion, apparently via a cyclic AMP-dependent pathway (290). These observations indicate that one might exploit lower organisms with their well-studied genetics to address many aspects of the transcriptional regulation of plant and plant virus genes.

(iii) Other promoters of plant pararetroviruses. Figwort mosaic virus (FMV) is a caulimovirus related to CaMV. The FMV equivalent of the 35S promoter has been characterized as a strong promoter in tobacco plants (137, 299). The equivalent of the 35S promoter with double enhancer has also been constructed from FMV sequences; in parallel transient-expression assays with *Nicotiana edwardsonii* protoplasts, this promoter appears to be 20 to 40% stronger than its CaMV counterpart (211), although FMV accumulates in many hosts to very small amounts per cell compared with CaMV (211).

Peanut chlorotic streak virus is a caulimovirus with wider host range than other known members of the group (277). Its genomic promoter, also called the 8.3-kb promoter (277), is comparable in strength to the FMV genomic promoter (135).

The genome of soybean chlorotic mottle caulimovirus contains, in addition to the two conventional caulimovirus promoters, a promoter-like sequence located upstream of ORF IV (145). When the three promoters were compared with the CaMV 35S promoter in transient-expression assays with tobacco protoplasts (56), the activity of a reporter gene expressed under the control of the soybean chlorotic mottle virus genomic promoter was fivefold higher than that of the CaMV 35S promoter (56).

Badnaviruses, the second group of plant pararetroviruses, are similar to caulimoviruses in replication strategy and in the sequence of several essential genes; however, the two groups are different in their mechanisms of gene expression (57, 285). Caulimoviruses infect only dicots, whereas different members of the badnavirus group infect either dicots or monocots. Badnaviruses can be transmitted by an insect vector but not by mechanical inoculation; cloned virus DNA can be introduced into a susceptible plant only by agroinfection (57). Such behavior often indicates that the virus is phloem limited. These observations prompted an investigation of the host and tissue specificity of badnavirus promoters that might account for the biological differences between two virus groups.

The first badnavirus promoter studied in some detail, the genomic promoter of commelina yellow mottle virus (CoYMV), was active in both dicot and monocot cells in transient-expression assays (217). In stably transformed maize callus, the CaMV 35S promoter with double enhancer and the CoYMV promoter had similar strength (218). In transgenic tobacco plants, the CoYMV promoter directed a high level of transgene expression in vascular tissue and in most types of tissues within the anthers (217). Tobacco is a nonhost plant for CoYMV; in a host plant, *Commelina diffusa*, CoYMV particles are found in mesophyll cells, and it should be expected that at least in this host, the CoYMV promoter would be active in other types of cells.

Deletion analysis of the CoYMV promoter revealed that most of its activity is confined to the −230 to +8 region relative to the transcriptional start and that the sequence −230 to −200 contains a transcriptional enhancer. The element responsible for vascular expression in tobacco was found at the −160 to −88 position. This sequence confers activity in vascular tissues to promoters that are normally inactive there (218).

The genomic promoter of another badnavirus, rice tungro bacilliform virus, is active mostly in phloem in rice and in transgenic tobacco plants (25), although it is able to direct strong transient expression in protoplasts from both hosts and nonhosts (52). Tissue specificity determinants are confined to sequences up to −169 from the transcription start, whereas the upstream sequence enhances the level of transcription through interaction with two groups of transcription factors (372). In addition, a downstream enhancer sequence of the rice tungro bacilliform virus promoter was detected by deletion analysis at positions from +8 to +83 (52).

Taken together, data on caulimovirus and badnavirus genomic promoters suggest that their organization is modular and that segments from different virus promoters may be combined with one another or with nonviral sequences to confer a desired pattern of expression in a particular host.

Additional control of transcription might be achieved by positioning caulimovirus promoters close to each other, as in the case of two promoters in CaMV (92). The 19S subgenomic promoter is weak; when compared in parallel transient-expression assays in protoplasts, the expression level of a reporter under the control of the 19S promoter is around 1% of the level of the same reporter controlled by the 35S promoter in both host and nonhost cells (92, 248). The 35S enhancer activates the 19S promoter when fused either immediately upstream of the 19S promoter or downstream of the reporter gene (92). The latter configuration is similar to the relative position of the two promoters observed in the circular caulimovirus genome (Fig. 1). Unexpectedly, sequences derived from the 19S promoter have been found to activate the 35S core promoter, although in the 19S promoter itself they could be deleted with little effect on gene expression (92). Thus, the two promoters in the caulimovirus genome seem to interact. It might be possible to achieve coordinated levels of expression of two foreign genes in plants by appropriate spatial arrangement of their caulimovirus-derived promoters.

(iv) Transcription termination signals in caulimoviruses. Both caulimovirus RNA transcripts are polyadenylated at their identical 3′ termini. Accurate processing of the 3′ termini of the eukaryotic mRNAs involves an endonucleolytic cleavage at a specific site and addition of a poly(A) sequence (26, 294, 353). Regulatory *cis* sequences required for these two processes vary in plants, yeasts, and mammals; the conserved AAUAAA site, ubiquitous in yeast and mammalian mRNAs, is also found in caulimovirus RNAs, although it is less common in plant messengers (294, 295, 353). This site is typically located 10 to 50 nt upstream of the RNA cleavage site, and its removal is usually deleterious for both cleavage and poly(A) addition. Commonly, additional *cis* sequences are required for efficient transcript cleavage, for determination of the correct cleavage point, and for poly(A) synthesis. These sequences are

located downstream of the AAUAAA motif in mammals; however, in plants, in plant viruses, and mammal viruses, they are found at various distances upstream of the AAUAAA motif (140, 295, 297, 353).

Recent detailed analysis of CaMV and FMV polyadenylation signals has revealed that the most important of the upstream *cis* sequences is the repetition of a short motif, for which the consensus sequence UUGUA has been proposed (286, 296). The AAUAAA sequence tolerates substitutions in CaMV (285) and is probably inactive in FMV, where the nearby UAUAAA element apparently serves as its functional equivalent (296). How these sequences interact with plant factors to ensure efficient formation of a polyadenylated virus RNA is unknown. However, termination signals derived from caulimovirus genomes are commonly used in plant gene vectors (see, e.g., references 262, 272, and 288).

The entire transcriptional termination signal of CaMV and probably of other caulimoviruses is located downstream of the promoter and is encountered by the transcription machinery twice during synthesis of the terminally redundant 35S RNA (295, 297). At the first encounter, recognition of the termination signal is reduced, allowing transcription to proceed and to yield the functional transcript. This occlusion of the termination signal is believed to be determined by its proximity to the promoter, which confers a low functional competence of the cellular transcriptional machinery to terminate transcription on the nascent RNA shortly after initiation (295–298). Interestingly, in some resistant hosts of CaMV, efficiency of premature termination at the upstream polyadenylation signal may be strongly increased, rendering infection abortive (298). Understanding of the mechanism of this process might allow one to construct transgenes that will be transcriptionally controlled by upstream premature termination signals, analogous to prokaryotic attenuators (148).

(v) Geminivirus transcription signals and *trans*-activator proteins. Geminiviruses are plant viruses with single-stranded (ss), circular DNA encapsidated in virions which resemble icosahedra fused in pairs. Replication of geminiviruses occurs in the nucleus by the concerted action of virus-encoded and cellular activities, presumably by a rolling-circle mechanism (29, 161, 186, 325). At least three groups of geminiviruses are currently defined (29, 336). Group I is composed of monocot-infecting, whitefly-transmitted viruses with narrow host ranges and monopartite genomes. Maize streak virus (MSV) and wheat dwarf virus (WDV) are the best-studied members of this group (Fig. 2). The sole member of group II is beet curly top virus, which has a monopartite genome, is leafhopper transmitted, and infects dicots from several dozen families. Group III members, as exemplified by tomato golden mosaic virus (TGMV) and African cassava mosaic virus, are dicot-infecting, whitefly-transmitted viruses with bipartite genomes (Fig. 2). During virus infection, ss DNA is converted into ds, circular, supercoiled intermediates (see the next section), from which virus transcripts are synthesized.

The two genomic components of the bipartite geminiviruses are termed A and B; only the A component is capable of autonomous replication in plant cells, whereas the B component provides proteins necessary for intercellular and systemic movement and, probably, also for nucleocytoplasmic export of virus DNA (95, 144, 230, 232, 240, 281, 328, 333). The genome of the monopartite geminiviruses can be regarded as an equivalent of the DNA A component, to which small genes required for intercellular movement are added (32, 336). These autonomously replicating entities range in size from approximately 2.6 to 3 kb and encode four to six proteins larger than 10 kDa. ORFs in geminivirus genomes are found in both the virion-encapsidated strand and the complementary strand (Fig. 2). Analysis of consensus initiation and termination sequences and transcript mapping revealed that the ca. 200-bp intergenic region contains promoters and other sequences that control initiation of transcription of virus genes. The smaller intergenic region contains AATAA motifs that might be the polyadenylation signals (370).

In the A component of bipartite geminiviruses, transcription of the virion-sense strand gives rise to a single RNA species. Its translation product is called the AV1 protein, for A-encoded, virion sense, or, alternatively, AR1, for rightward expression (28, 29, 230, 336). This protein is the capsid protein. In monopartite geminiviruses, rightward expression results in the synthesis of one or two major transcripts that direct translation of two or three proteins, virus capsid protein (V2) among them. Leftward expression in bipartite geminivirus DNA A starts at various positions either within the common region or further downstream (Fig. 2) and terminates in the small intergenic region. The leftward transcripts are thus 3′ coterminal. Protein products of these transcripts are AC1 (AL1), the only virus-encoded protein that is essential for virus DNA replication by the rolling-circle mechanism (see below); AC2 (AL2), the transcriptional activator of expression of several other virus genes; and AC3 (AL3), an auxiliary protein that increases virus DNA synthesis. In monopartite geminiviruses, only a counterpart of AL1, called the C1-C2 protein, is clearly defined.

It has been shown that in MSV, V1 and C1-C2 transcription is directed by two head-to-head-oriented core promoters with TATA-like boxes; the virion-sense core element could be activated in transient-expression protoplast assays by the enhancer sequence from the CaMV 35S promoter (101). In addition, a 122-bp sequence between the two core elements serves as a transcriptional enhancer, probably bidirectionally (102). Spread of several monopartite geminiviruses in monocots is thought to be limited to the cells of vascular bundles, whereas virions of MSV have also been observed in mesophyll cells (32, 33, 335, 336). It is not known whether these differences reflect different tissue specificities of monopartite geminivirus promoters.

Several regulatory mechanisms involving interaction of virus DNA component A-encoded proteins with virus promoters in bipartite geminiviruses have been recently revealed (142, 327, 328). The AL2 gene product of TGMV has been shown to activate transcription of a reporter gene from AR1 and BR1 promoters in leaf discs and in protoplasts (327). Moderate activation of additional virus promoters was also observed in African cassava mosaic virus (142). In a similar type of experiment, the weak promoter of the capsid protein gene in a monopartite geminivirus, chloris striate mosaic virus, was shown to be enhanced by the capsid protein itself, as well as by the C1-C2 replication protein (374).

In transgenic tobacco plants, the AR1 promoter of TGMV directed reporter gene expression only in the cells of vascular bundles and in meristems; however, when the AL2 protein was provided by virus infection or by a cross with another transgenic plant, the AR1 promoter was strongly activated in mesophyll cells (27, 289).

AL1 protein is the early virus protein essential for replication. It is thought to be required in only moderate amounts (328). It has been shown in transient-expression assays that expression of a reporter gene under the AL1 promoter of TGMV is decreased in the presence of the AL1 protein itself. Thus, synthesis of the AL1 protein in TGMV is autoregulated at the level of transcription, most probably involving direct interaction of the protein with a distinct *cis* element recently found just upstream of the AL1 promoter (93).

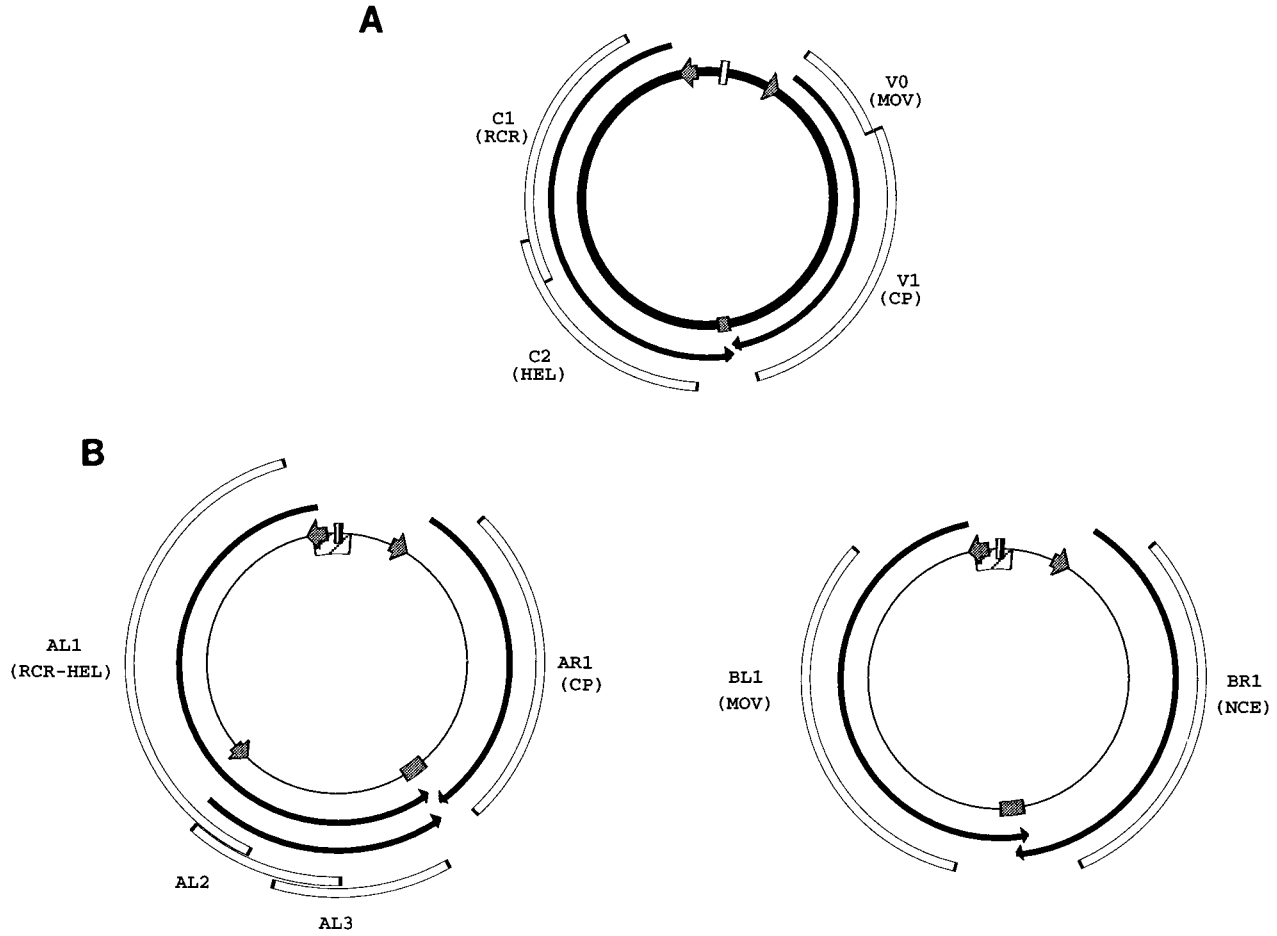

FIG. 2. Organization of geminivirus genomes. (A) MSV (geminivirus group I). (B) TGMV (geminivirus group III). The circle indicates the monomer of virus genomic DNA. Virion DNA is ss DNA. The single component of the MSV genome is of 2.8 kb, and the two components of the TGMV genome are of 2.6 kb each. Black arrows represent major virus RNA transcripts. Shaded arrowheads indicate the location and orientation of virus promoters. Shaded boxes show the position of virus terminators. Striped boxes in the TGMV genome indicate the position of the shared DNA region. Black boxes indicates the 9-nt sequences implied in the initiation of the rolling-circle DNA replication. Open boxes represent major virus proteins. The names of the proteins are indicated, and known or putative functions of the proteins are given parenthetically. CP, capsid protein; HEL, putative helicase domain; RCR, rolling-circle replication initiator; MOV, cell-to-cell movement protein; NCE, protein required for nucleocytoplasmic export of virus DNA.

Recently, promoter activity was detected in a DNA segment derived from the cloned genome of the coconut foliar decay virus, a small "circo-like" virus with ss circular DNA of 1,291 nt (281a). This promoter directed phloem-specific expression of the GUS reporter gene in transgenic tobacco (281a). Interestingly, although coconut foliar decay virus is likely to replicate by the rolling-circle mechanism, as do geminiviruses (186, 282; also see the next section), a distinct feature of its promoter is a 52-bp stretch that shows 70% identity to a region within the CoYMV promoter. It is possible that this sequence is required for interaction with certain phloem-specific transcription factors (281a).

Summary. For more than a decade, elements that control transcription in plant DNA viruses have been of utmost importance for the development of plant transformation technology. The 35S promoter of CaMV was among the first to satisfy the demand for a strong constitutive promoter that is active in many plant species. Derivatives of this promoter that are even stronger than the wild type are also widely used. However, it seems likely that the search for stronger and stronger constitutive promoters is over, given the rapidly accumulating data on targeted degradation of overproduced RNA and on the other mechanisms of gene silencing (67, 103, 138, 229, 348). More effort toward development of the controlled promoters that can be switched on in response to an effector or in a tissue-specific manner is now justified. As should be evident from the above, plant virus sequences will probably be valuable in this endeavor.

DNA-directed DNA synthesis. (i) Features of geminivirus replication. Virion DNA of geminiviruses is ss DNA. Upon entry into the host cell nucleus, it is converted into the ds form by host enzymes. In monocot-infecting geminiviruses, a DNA fragment of approximately 80 nt is tightly bound to the virion DNA (85, 151). This fragment is able to prime virus DNA synthesis in vitro and is complementary to the small intergenic region which has been shown genetically to contain a *cis* element required for efficient complementary strand synthesis (174). In bipartite geminiviruses, *cis* sequences required for complementary-strand synthesis are most probably located within the common region and no putative primer DNA is found in the virion (230, 336).

After conversion into a ds molecule, geminivirus DNA is transcribed by host enzymes and virus proteins are synthesized. The AL1 (AC1) protein of bipartite geminiviruses and a ho-

mologous C1-C2 product of monopartite geminiviruses are crucial for virus replication (2, 95, 144, 301, 344). Computer-assisted sequence comparisons revealed that these proteins contain conserved sequence motifs indicative of two important replication-associated functions. The C1 proteins, as well as the N-terminal halves of AL1 proteins, contain three motifs shared with a group of Rep proteins encoded by the ss DNA plasmids of gram-positive bacteria; in bacterial proteins, these motifs are essential for the endonucleolytic cleavage of DNA that initiates its replication by the rolling-circle mechanism (161, 186). The C2 proteins and the C-terminal halves of AL1 proteins contain motifs that are found in diverse DNA and RNA helicases that are involved in many aspects of nucleic acid turnover, including unwinding of the ds replication intermediates (133).

In accord with the postulated rolling-circle replication mechanism of geminivirus DNA, a *cis* element that consists of a conserved inverted repeat that may form a hairpin-loop structure has been identified in the large intergenic region (197). Within the loop, there is an invariant 9-nt sequence that resembles the well-studied endonucleolytic cleavage sites found in the origins of replication of bacteriophages and plasmids (186, 325). Analysis of recombinants between wild-type and artificially mutated genomes in African cassava mosaic virus revealed that the nick is indeed introduced in the virion-sense DNA strand within the conserved sequence TAATATT*AC (the asterisk indicates the position of the nick [323]).

Direct evidence compatible with the rolling-circle mechanism of geminivirus replication has been obtained (325). Release of the unit-length DNA genome from tandemly repeated copies of cloned DNA upon agroinfection revealed that whenever the two copies of the hairpin-loop sequence were present in the construct, the predominant genotype in the progeny consisted of the sequence between the hairpins (325). The geminivirus AL1 protein is believed to perform ss DNA cleavage at its cognate site in the hairpin with the circularization of the released DNA fragment (144, 153, 333). This site overlaps with but can be mutated separately from the AL1 transcriptional activator element (93). It has been concluded that the origins of replication for both DNA strands, together with the AL1 protein, constitute the set of essential elements that should be a part of any geminivirus-based replication system (29). Replicating vectors based on geminivirus genetic elements are discussed in more detail below, in the section dealing with the virus vectors.

Release by the AL1 protein of the ss DNA segment from an integrated DNA flanked by two geminivirus hairpin-loops results in formation of a long ss gap that is filled by the host repair enzymes. This molecule then serves for further rounds of release-repair. This might be exploited to amplify a segment of DNA flanked by the appropriate AL1 recognition sites. Further progress in understanding of geminivirus replication mechanisms might allow one to uncouple this process from the later stages of the virus life cycle, thus creating nonautonomous, high-copy-number DNA replicon.

RNA-directed RNA synthesis. The vast majority of plant viruses do not have a DNA stage in their life cycle. The RNA genomes encode proteins that mediate RNA-dependent RNA synthesis in concert with the host-encoded factors. In particular, all sequenced nondefective RNA viruses code for a polypeptide that is believed to possess an RNA-dependent RNA polymerase activity, also referred to as the polymerase or polymerase-like protein. To enable specific and efficient amplification of virus RNAs, these enzymes form complexes with host-encoded factors and with other virus-encoded proteins and interact with the *cis* elements found on virus RNAs. Knowledge of the structure and function of these components would permit the construction of RNA replicons which multiply in plants extrachromosomally.

Among RNA viruses, the largest and best-studied group is composed of the viruses that have positive-strand virion RNA. Viruses with such a genome strategy constitute more than 70% of the known plant viruses (105, 214). Other groups of plant RNA viruses, those that possess negative-sense RNA, ambisense RNA, and ds virion RNA, are less well studied in molecular terms, and infectious RNA copies of their cloned genomes are not yet available. We will not discuss replication of these groups of plant viruses, although recent advances in studies of the related viruses in animals and fungi are noteworthy (see, e.g., references 242 and 361).

(i) Viral polymerase. The unexpected sequence similarities linking plant and animal viruses were first observed about 10 years ago (132, 146, 172) and have now been investigated in some detail (see, e.g., references 83, 128, 184, and 185). The basic concept now seems to be well established that many positive-strand RNA viruses with diverse modes of genome organization and with hosts as different as animals and higher plants share related protein domains essential for expression and replication of their RNAs. Comparison of protein sequences encoded by RNA viruses culminated recently in reconstruction of the putative common ancestor of all known positive-strand RNA viruses (185). This analysis relies largely on the identification of several proteins that are thought to be the components of the virus-encoded RNA replication complex.

The most highly conserved protein found in all autonomously replicating RNA viruses is the core subunit of the RNA-dependent RNA polymerase. According to the latest detailed comparison (184), eight conserved sequence motifs can be delineated in different members of this vast supergroup of virus proteins, with three motifs (IV, V, and VI) shared by all polymerase-like proteins. It has been shown by site-directed mutagenesis that these three motifs are essential for polymerase activity encoded by an animal picornavirus, the encephalomyocarditis virus (300). Polymerase-like proteins of RNA viruses form three lineages, each including enzymes of both animal and plant viruses (185).

Most of the positive-strand RNA viruses with genome sizes of more than 6 kb encode another protein that is thought to be a component of the RNA replication complex. This protein contains several conserved sequence motifs characteristic of the nucleic acid-binding proteins and, more specifically, of RNA helicases (131–133, 185). These proteins have been implicated in an energy-dependent unwinding of the RNA duplex upon RNA synthesis and possibly upon translation as well (131–133, 183, 322). RNA-dependent ATPase and helicase activities have been demonstrated for the CI protein of a plant virus, the plum pox potyvirus (191, 192). Together with data on site-directed mutagenesis (225, 226, 332, 360), this evidence establishes an essential role for the helicase-like activity in RNA virus replication, notwithstanding the apparent lack of virus-encoded helicase-related proteins in RNA viruses with smaller genomes.

Positive-strand RNA virus helicases belong to three distinct superfamilies; each of the three also includes helicases of DNA viruses and helicases encoded by cellular genomes (133). Interestingly, preferential combinations of the type of RNA polymerase and the type of RNA helicase in a given virus genome seem to exist, resulting in three, rather than nine, major divisions of positive-strand RNA viruses (185).

In many positive-strand RNA viruses, including a variety of plant viruses, genomic and subgenomic RNAs are capped (i.e.,

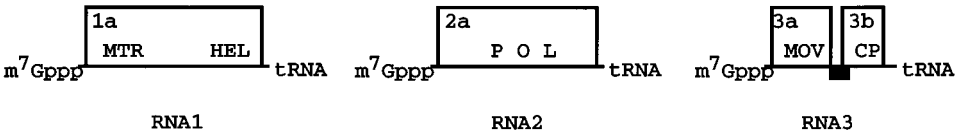

FIG. 3. Organization of the genome of BMV (bromovirus group). Three genomic RNA components of BMV (RNA1, RNA2, and RNA3) are indicated by horizontal lines. Individual ORFs (1a, 2a, 3a, and 3b) are shown by open boxes. The sequence that serves as the subgenomic promoter on the complementary strand of RNA3 is indicated by the black box. m^7Gppp, cap structure at the 5′ termini of genomic RNAs; tRNA, tRNA-like structure at the 3′ termini of genomic RNAs; MTR, putative methyltransferase domain; HEL, putative helicase domain; POL, putative RNA-dependent RNA polymerase; MOV, cell-to-cell movement protein; CP, capsid protein.

possess a methylated guanosine residue linked to the 5′ end of the RNA by a 5′-5′ phosphodiester bond). It might be expected that because replication of positive-strand RNA viruses is cytoplasmic, the cellular capping enzyme will not be available for modification of virus RNAs. It has been shown by genetic and biochemical analysis that a particular domain in the nonstructural protein of animal alphaviruses codes for methyltransferase activity (220, 221). Related domains were identified in many plant virus nonstructural proteins by computer-aided sequence analysis (287).

In some virus groups, activities required for RNA replication also include proteases that release polymerase and helicase from the polyprotein precursors (88, 185). In a plant virus, alfalfa mosaic virus (AlMV), replication can be initiated only in the presence of the coat protein or its amino-terminal segment (12). In plant viruses from several groups, wild-type levels of replication are achieved only in the presence of the functional coat protein (357) or when aided by a protein covalently linked to the 5′ termini of the genomic RNAs (VPg [223, 231]). The arrangement of replication-associated domains in the genomes of representative plant viruses is shown in Fig. 3 through 7.

The best-studied examples of plant virus RNA replication enzymes include RNA replication complexes of brome mosaic bromovirus (BMV) and cucumber mosaic cucumovirus (CMV) (54, 149, 268). Both include two virus-encoded polypeptides with three putative activities. The 1a protein contains methyltransferase and helicase domains, and the 2a protein codes for RNA polymerase (Fig. 3). In the case of BMV, the two proteins form a complex in vitro and the protein segments required for the interaction of BMV 1a and 2a proteins have been mapped (54). The highly purified, biochemically active complex of BMV RNA polymerase contains a host-encoded polypeptide related to a component of translation elongation factor 3 (268). The replication complex of BMV is able to direct synthesis of viral negative-strand virus RNA on a positive-strand template and also of subgenomic RNA 4 on the negative strand of RNA 3. The purified replication complex in CMV is probably similarly organized and is capable of synthesizing both positive and negative strands of CMV RNAs (149).

That the host components of plant virus RNA replicases are conserved in a variety of organisms is convincingly illustrated by the ability of yeast cells to support replication of BMV RNA 3 when the 1a and 2a proteins of the virus are expressed in these cells (165).

Tobacco plants transformed with BMV or AlMV 1a and 2a proteins were able to replicate the homologous RNA 3 (227, 347). Recently, several mutants with mutations in the putative RNA polymerase domain (NIb protein) of tobacco etch potyvirus that were unable to replicate in vivo but propagated to near wild-type levels in transgenic tobacco plants expressing the functional NIb have been characterized (205). In principle, heterologous RNAs should be amplifiable in such plants, provided that they carry replication signals recognized by virus enzymes. In the next two sections, the structure of these signals is considered.

(ii) Terminal and internal recognition sites. About 30 years ago, it was found that the 3′ end of the genomic RNA of turnip yellow mosaic tymovirus (TYMV) becomes covalently linked to valine in the presence of an enzymatic activity from bacterial extracts (18, 257). This observation was followed by the discovery of amino acid acceptor properties, as well as other tRNA-like features, in the 3′-terminal regions of RNAs of other plant viruses, i.e., tymoviruses, tobamoviruses, hordeiviruses, bromoviruses, and cucumoviruses. Mutational analysis revealed that loss of aminoacylatability at the 3′ termini invariably abolished virus RNA synthesis, unless second-site suppressor mutations restored both functions (91, 222, 224, 338). Although the functional role of aminoacylation in plant virus RNAs is still unclear, the 3′-terminal tRNA-like structures are probably the best-studied class of *cis* elements required for initiation of virus RNA synthesis (100, 124).

Although plant tRNA-like structures and cellular tRNAs share additional structural features (occurrence of an anti codon and, in some cases, of the TΨC loop) and functional properties (interaction with tRNA nucleotidyl-transferase, RNase P, and translational elongation factors [141, 169, 207]), the plant virus sequences cannot be folded into a cloverleaf structure. Instead, a conformation similar to the L shape of tRNA can be achieved, if pseudoknotting is allowed (100, 124, 259). On the whole, these tRNA-like structures are larger than the canonical tRNAs (100).

A variety of mutants or deletion derivatives of genomic RNAs that are amplified only in the presence of the replicative proteins provided in *trans* by the coreplicating nondefective genomes have been constructed in tobamoviruses, bromoviruses, and cucumoviruses (30, 106, 108, 269). This approach was used to assess the size of the 3′-terminal sequences required for RNA replication. It has been shown that the intact tRNA-like structure (134 nt) is sufficient for synthesis of BMV minus strand by virus-specific replicase preparations in vitro (222) but that efficient replication of virus RNA in vivo requires additional upstream sequences (106). In several viruses, one or more pseudoknots can be predicted for these regions and might be essential for some aspect of RNA replication (259). In tobacco mosaic tobamovirus (TMV), at least 900 3′-terminal nucleotides, including several potential pseudoknots, were needed for replication at an appreciable level (269).

In many groups of plant RNA viruses, the 3′ end of the genome does not display tRNA-like properties. Requirements for the interaction of such RNAs with their replication enzymes are not well understood. In one study, derivatives of the polyadenylated RNAs 3 and 4 of beet necrotic yellow vein virus that could be replicated in the presence of wild-type RNAs 1 and 2 of this virus were constructed (171). It was shown that efficient RNA replication required the 123 nt preceding the poly(A) tail at the 3′ end of RNA 4 but only 69 nt upstream of

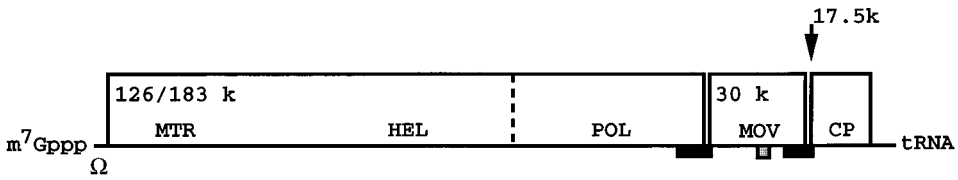

FIG. 4. Organization of the genome of TMV (tobamovirus group). The 6.4-kb genomic RNA of TMV is indicated by the horizontal line. Individual ORFs (126/183k [126 and 183 kDa], 30k [30 kDa], and 17.5k [17.5 kDa]) are shown by open boxes. The position of the leaky termination codon within the 126- and 183-kDa gene is shown by a broken line. Black rectangles indicate the positions of two subgenomic promoters on the complementary RNA strand. The shaded box indicates the position of the origin-of-assembly sequence. The vertical arrow indicates the site where foreign genes followed by the subgenomic promoter of related odontoglossum ringspot tobamovirus are inserted into the TMV-derived expression vector. m^7Gppp, cap structure at the 5′ terminus of genomic RNA; Ω, 5′-terminal translational enhancer; tRNA, tRNA-like structure at the 3′ terminus of genomic RNA; MTR, putative methyltransferase domain; HEL, putative helicase domain; POL, putative RNA-dependent RNA polymerase; MOV, cell-to-cell movement protein; CP, capsid protein.

the poly(A) at the 3′ end of RNA 3. The 69-nt segment is conserved in RNAs 1, 2, and 3, giving an estimation of the size of a minimal 3′-terminal *cis* sequence for replication of other RNA segments in this virus (125, 171).

Elements involved in the synthesis of positive RNA strands in bromoviruses have been characterized. Interestingly, the structure of these elements again suggests that an intimate link may exist between cellular tRNAs and virus RNA replication. In the bromoviruses, short sequence elements that resemble the internal control regions of the promoters of cellular tRNA genes have been found close to the 5′ termini of the positive-strand RNAs (213, 260). One of the motifs, the ICR2-like motif, was also found in many other plant RNA viruses (260). Mutational analysis indicated that these sequence elements, as well as the proximal stem-loop element, are the integral parts of the promoter for virus positive-strand synthesis (260). Studies of the artificially deleted RNA replicons in bromoviruses, cucumoviruses, tobamoviruses, and beet necrotic yellow vein virus have demonstrated that the 5′-terminal sequences required for efficient RNA replication in vivo are generally smaller than the 3′-terminal elements, from only 23 nt in one of the TMV deletion variants to 92 nt in CMV RNA 3 (30, 171, 249, 269).

The internal portions of some plant virus RNAs were also found to be essential for genomic RNA amplification. RNA 3 in related bromoviruses and cucumoviruses is bicistronic (Fig. 3). An intercistronic segment of 100 to 160 nt is required for positive-strand RNA synthesis in both groups (30, 106). In BMV, this region contains additional copies of the ICR2 motif (260, 261). It is not known whether intercistronic location of these sequences is essential for their activity or whether they can be relocated closer to an RNA terminus.

The main conclusion from these observations that is relevant to genetic engineering is that the *cis* sequences essential for RNA replication are compact, generally not exceeding 1,000 nt. Thus, substantial portions of most plant RNA virus replicons can be replaced by heterologous sequences to become amplifiable by virus replicative machinery provided in *trans.*

A note of caution should be sounded, however, because replication of many plant and animal RNA viruses appears to be *cis* preferential. Numerous mutated derivatives of TYMV, of cowpea mosaic comovirus (CPMV), or of tobacco vein mottling potyvirus could not be efficiently replicated in *trans* by a wild-type helper virus (305, 346, 359). Similar results were observed in the animal poliovirus (241). Interestingly, the genome of CPMV is bipartite, with replication functions encoded on the RNA B. Hence, replication of the other genome component, RNA M, is driven in *trans*; accordingly, deletion derivatives that replicate in the presence of wild-type RNA B can be prepared from RNA M but not from RNA B itself (346). It has been speculated that the replication complex of many viruses is assembled, possibly cotranslationally, on the same RNA molecule from which it has been expressed (359). This might represent a proofreading mechanism that reduces the possibility of propagation of nonfunctional deleted genomes (241). *cis* preference seems to be especially common in plant and animal viruses that employ proteolytic processing of their polyproteins (241, 359). Future investigations will establish whether these two phenomena are indeed related. Although the *cis* preferences of some replicative complexes might limit the use of disarmed virus replicons, this limitation might not be universal (compare the examples of the closely related tobacco vein mottling virus and tobacco etch virus [TEV] above).

Recently, a remarkable interaction between replication and translation has been discovered in cell-free bacterial lysates supplemented with the replication protein of an RNA bacteriophage (292). This system is known to support both replication and translation of heterologous RNAs if the latter are tailed with the termini of phage RNA. When the system was programmed to direct coupled replication and translation, the level of reported gene expression was indicative of a synergistic, rather than an additive, interaction between the two processes (292). Thus, autonomous cytoplasmic replication of an RNA messenger might be superior to its nuclear transcription because of the possibility of synergistic interaction with its own translation machinery.

(iii) Subgenomic promoters. Many positive-strand RNA viruses, and numerous groups of plant viruses in particular, express their 5′-distal genes from subgenomic versions of their genomic RNAs. It has been established that these RNAs are synthesized by virus RNA replicase that recognizes the genome-length minus-strand RNA internally (107, 224). An enzyme preparation from BMV-infected barley leaves directs synthesis of both minus-strand RNA and subgenomic RNA in vitro, although it is not known whether these processes and synthesis of positive-strand RNA require an identical replicative complex in vivo (222, 224, 268).

In addition to positioning of the otherwise silent cistrons close to the 5′ ends of their RNA templates, subgenomic RNAs serve to control a temporal pattern and the amount of synthesized proteins. TMV expresses two of its four known proteins via subgenomic RNAs (Fig. 4). Of these, the longer subgenomic RNA, which directs synthesis of the 30-kDa movement protein, is synthesized in small amounts transiently at an early stage of infection. In contrast, the amount of the shorter subgenomic RNA for the virus capsid protein increases linearly, reaching high levels late in infection (200, 201). Fusion of a heterologous gene in an RNA virus-based replicon to the appropriate subgenomic promoter might achieve a desired pat-

tern of gene expression (e.g., low and transient versus high and constitutive).

The structure of the promoters that direct synthesis of subgenomic RNA 4 from the minus strand of RNA 3 in bromoviruses has been investigated in some detail (106, 107, 249, 319). In BMV, inefficient but correctly initiated synthesis of RNA 4 occurs on the RNA 3 negative strand when the sequences from −20 to +16 relative to the initiation site are present (107). For a high level of RNA 4 synthesis, additional sequences are required. These consist of the $A_{16–22}$ tract, thought to be important for the appropriate juxtapositioning of the other sequence elements rather than for direct recognition of the enzyme (107, 319), and of at least 36 nt, but not more than 57 nt, of further upstream sequences that contain partial repeats of the motifs found in the core −20 promoter (107). Organization of the RNA 4 promoter in cowpea chlorotic mottle bromovirus is apparently similar, although the essential sequences upstream of the oligo(A) tract are smaller and simpler in this virus than in BMV (249). Motifs resembling the BMV subgenomic promoter are also found at equivalent positions in related cucumoviruses and alfamoviruses (107).

The structure of the subgenomic promoters in other groups of viruses has not been well studied. In the diverse group of luteoviruses, an ACAAA motif is often found at the vicinity of the initiation sites for subgenomic RNAs (223). It has been speculated that this motif or its complement might be a high-affinity polymerase-binding site (223).

Despite poor understanding of the structure and properties of plant virus subgenomic promoters, arbitrarily chosen segments upstream of the start codons of some 5′-distal virus genes were found to be good subgenomic promoters in vivo (84, 201). In many cases, the activity of subgenomic promoters is increased when the distance between the promoter and the 3′ end of genomic RNA is decreased (30, 60, 107).

Subgenomic promoters from plant viruses might be used to develop a system for virus-induced gene expression. An antisense RNA with a plant virus subgenomic promoter at the 5′ terminus would be translationally silent. In the presence of the replicating virus in the same cell, a cognate RNA replication enzyme would recognize the subgenomic promoter and would synthesize the sense strand of the transgene. This approach has recently been used to express an antisense strand of a bacterial toxin RNA in transgenic tobacco. The transcript contained the subgenomic promoter of the capsid protein of potato virus X (PVX). Upon virus infection, a complementary coding strand of the transgene was synthesized, resulting in toxin expression, induced cell death, and inability of the virus to spread efficiently from the sites of initial infection (10).

Translational Control in Plant Viruses

Genomic RNAs of positive-strand RNA viruses, including plant viruses, were among the first eukaryotic mRNAs that became available in large amounts. A significant effort was undertaken to study their translation in vitro and in vivo, with the aim of understanding the cellular translational mechanisms. Whereas viruses have certainly contributed to a better knowledge of translational regulation in eukaryotes, it should be noted that many plant virus RNAs might not be altogether adequate as model systems. Indeed, whereas the overwhelming majority of eukaryotic mRNAs have capped 5′ ends and poly(A) tails at the 3′ ends, the RNAs of plant viruses often lack one or both. It is often due to the presence of the alternative terminal structures that virus templates exhibit high translational efficiency.

In another example of disparity with the eukaryotic translational rules, plant (and animal) RNA viruses often use the same mRNA for expression of several separate or overlapping polypeptides. Mechanisms may include a modification of the ribosome-scanning model that will allow the use of alternative AUG initiator codons (leaky scanning), RNA recoding (translational frameshifting and leaky termination), and a particular protein-mediated mechanism of polycistronic translation in caulimoviruses. Additionally, a mechanism of internal ribosome entry, used by many viruses for expression of their single polyprotein, can be adapted to create artificial polycistronic mRNAs. Virus sequences that control these alternative regulatory mechanisms might facilitate the engineering of whole metabolic pathways, in which coordinated expression of several proteins is needed.

Translational enhancers. In positive-strand RNA viruses, genomic RNAs are translated early upon infection, even though cellular templates are prevalent. Features that increase translational efficiency or template specificity of such mRNAs are essential for virus competitiveness.

Indeed, the 68-nt leader of TMV genomic RNA and the 36-nt leader of AlMV subgenomic RNA 4 were the first plant RNA 5′ untranslated regions (UTRs) in which translational enhancement capacity was discovered (119, 166). Since then, a number of properties of plant virus 5′ translational enhancers have been investigated, including the role of the cap structure, interaction with ribosomes, requirement of translation initiation factors, and differential effect on heterologous gene expression in various cell-free systems and in vivo (64, 115, 116, 121, 262, 318, 337). The actual degree to which translation is enhanced by a given leader varied greatly in different investigations, reflecting different properties of commonly used cell-free translation systems and of plant protoplasts derived from different species. Extra nucleotides appearing at the 5′ ends as a result of engineering complicated the analysis (see reference 318 for a critical evaluation of early results). Generally, the output of a protein product translated from RNAs with plant virus translational enhancers is severalfold to a few dozen-fold higher than from a messenger with a random leader (318).

The best-studied translational enhancer is derived from the 6,400-nt genomic RNA of TMV. This RNA is capped at the 5′ end and has a tRNA-like sequence at the 3′ end. It is the messenger for translation of two 5′-coterminal, replication-associated proteins of 126 and 183 kDa (Fig. 4). The leader, also known as the Ω sequence, consists of 67 to 70 nt depending on the TMV strain and lacks G residues between the first G after the cap structure and the G in the AUG initiator codon of the 126- and 183-kDa proteins (318) (Fig. 4). Sequence comparison between strains of TMV revealed two conserved motifs within the leader, an 8-base direct repeat reiterated twice or three times and a 25-base $(CAA)_n$ tract. Together, these two elements account for up to 72% of the leader (121).

To dissect this structure, deletion derivatives were synthesized and cloned upstream of the reporter genes, luciferase and GUS, in plant expression cassettes. Transcribed and capped mRNAs were electroporated into plant protoplasts, and the expression of reporter genes was analyzed. The intact Ω sequence or its derivatives does not affect the stability of mRNA or interfere with the recognition of the AUG codon context (117, 119). However, the translation rate of the luciferase reporter with the Ω sequence was more than 10-fold higher over the lifetime of the template than was that of the same reporter with a random leader (121). Within the first few minutes after electroporation, the increase was up to 90-fold higher. The major enhancing effect was exhibited only when the leader was not shorter than 44 nt, i.e., within the length limits from 44 to 74 nt (wild type). Enhanced expression was

dependent on a combination of one CAA element and one 8-nt repeat or of two CAA elements. Reiteration of either of the two elements further improved expression (121). Although the mechanism of the translational enhancement by the Ω sequence or by its derivatives is not understood, the enhancement might be favored by the apparent lack of secondary structure in this RNA segment and is thought to involve interaction of the CAA repeats with a specific cellular protein (115).

An interesting facet of the TMV translational enhancer is that it is also active on uncapped templates in vitro and in vivo. Moreover, in prokaryotic cells, it is able to substitute for the prokaryotic cell-specific Shine-Dalgarno sequence in the initiation of translation (118).

Another plant virus translational enhancer, the 36-nt leader from AlMV RNA 4, does not contain any recognizable sequence motif that would serve as a core enhancer element, and its enhancing capacity is strongly dependent on the presence of the cap structure. In the absence of the cap, an enhancing effect is observed only with the addition of large amounts of eukaryotic initiation factor 4F (115).

In a third example of translational enhancement by a capped 5′ UTR of a plant virus, the leader of PVX (a potexvirus) genomic RNA was examined (262, 337). The entire 83-nt sequence enhances expression of a GUS reporter gene several-fold in vitro and in vivo. It consists of the α segment of 41 nt that is G free and is presumed to be unfolded and the 42-nt β segment with the potential of forming some secondary structure. Enhancement of the authentic PVX 5′ proximal cistron was stronger when the β segment was deleted from the leader. A majority of the α segment was also dispensable for the enhancement. The only element essential for enhancer activity was the CCACC pentanucleotide within the α segment. This element may be implicated in interaction with the complementary 3′-terminal sequence in 18S rRNA (337).

Specific proteins covalently linked to the 5′ termini of the genomic RNAs have been demonstrated for the members of several groups of plant viruses, e.g., potyviruses, luteoviruses, comoviruses, and nepoviruses. Given that potyviruses alone constitute about 20% of known plant viruses (214), this version of the uncapped mRNA is common among plant RNA viruses, along with the cap-independent mode of RNA expression. However, very little is known about the properties of naturally uncapped 5′-UTRs. In one better-studied example, the 144-nt leader from TEV genomic RNA was cloned upstream of the GUS reporter gene and the transcripts were translated in a cell-free system (44). Expression was shown to be cap independent, i.e., it neither required cap structure nor was inhibited by cap analogs. Addition of this leader to the plant virus expression cassette resulted in at least a 20-fold increase in translation rate in tobacco protoplasts and in a 5-fold enhancement in transgenic tobacco (44).

An example of a virion RNA with an unmodified 5′ end is the satellite tobacco necrosis virus (STNV), a simple genome that exploits the replicative machinery provided by the helper, tobacco necrosis virus. STNV RNA comprises a 29-nt 5′-UTR, a single 591-nt cistron coding for the capsid protein, and a ca. 620-nt 3′-UTR. STNV RNA is very efficiently translated in vitro; removal of 12 nt from the 5′ UTR decreases translation, but capping of this truncated transcript restores its high translational rate (334).

All of the above examples show the importance of the sequences upstream of the first functional AUG codon for the efficient translation of virus mRNAs. Interestingly, it has been found that the sequences immediately downstream of the initiator codon, i.e., within the first ORF, can also have a positive effect on translation (109). In Sindbis virus, an animal alphavirus, the secondary structure at the beginning of the cistron encoded by the 26S subgenomic RNA appears to improve the translational capacity of the ribosomes in virus-infected cells (109).

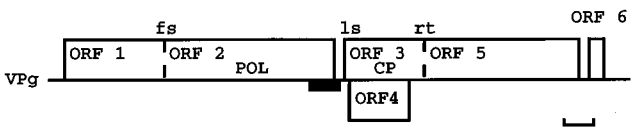

FIG. 5. Organization of the genome of BYDV-PAV (alloluteovirus group). The 6.4-kb genomic RNA of BYDV-PAV is indicated by the horizontal line. Individual ORFs (ORFs 1 to 6) are shown by open boxes. Two leaky termination codons that are recoded by the frameshift (fs) or by the readthrough (rt) are shown by broken lines. The black rectangle indicates the position of the subgenomic promoter on the complementary RNA strand. The horizontal bracket indicates the position of the downstream *cis* element required for efficient cap-independent initiation of translation of the genomic RNA. VPg, covalently linked protein at the 5′ terminus of virus RNA; POL, putative RNA-dependent RNA polymerase; CP, capsid protein; ls, initiation of translation of the subgenomic RNA at two different initiator codons by leaky scanning.

The primitive model, in which translation is largely dependent on the 5′ sequences on an mRNA whereas the 3′ segments are at best involved indirectly, e.g., through conferring stability to the RNA template, is currently under further scrutiny with the advent of data on distinct 3′ elements specifically operating at the translational level.

In TMV genomic RNA, a 105-nt tRNA structure at the 3′ end is involved in replication but not in translation whereas the preceding 72-nt pseudoknot region is required for efficient translation. Reporter genes that are fused to both the 5′-enhancer and 3′-pseudoknot region are translated more efficiently than RNAs with the 5′ enhancer alone. Activity of the 3′ region is not limited to stabilization of RNA levels, and the 3′ sequence is inactive when the template is not capped (114, 120, 198). How the termini of TMV RNA interact to achieve this synergistic enhancement is unclear. Sequences within the long 3′-UTR in STNV RNA were also shown to enhance translation with their cognate 5′-UTR; in this case, direct base pairing between the two segments might be involved (63, 334). The 3′-UTR of AlMV RNA 4 apparently enhances translation by facilitating the loading of this RNA onto ribosomes (291).

In barley yellow dwarf virus strain PAV (BYDV-PAV), genomic RNA is translated in a cap-independent manner (223). Neither the structure of the 5′ end of BYDV-PAV RNA nor the mechanism of the cap-independent translation is known in this instance. However, a far downstream *cis* element that is necessary for translation of the uncapped RNA in vitro but has little effect on translation when the RNA is artificially capped has been characterized (223) (Fig. 5).

These observations emphasize the importance of downstream elements in the processes occurring near the 5′ end of an mRNA during its translation. Incorporation of the 3′-UTRs of plant viruses into expression cassettes may be useful to optimize regulatory properties of their 5′ counterparts.

Expression of multiple proteins from one mRNA. (i) Internal ribosome entry. The mechanism that enables some virus mRNAs to be translated in a cap-independent manner is not understood. It is recognized that the VPg at the 5′ terminus of the genome in animal picornaviruses is not essential for the initiation of translation; instead, initiation is achieved via interaction of a ribosome and a distinct *cis* element, referred to as the internal ribosome entry site (IRES), within the virus 5′-UTR (3, 253, 256, 322, 369). When positioned internally between two cistrons on a chimeric mRNA, IRES elements of

picornaviruses promote initiation of translation of the downstream cistron, presumably by direct binding of 40S ribosomal subunits (253, 322, 369). This property of IRES is used in animal expression vectors, in which it enables expression of several genes of interest along with selectable markers as a single transcriptional unit (8).

A 5′-terminal VPg attached to plant virus RNAs has been found in potyviruses, comoviruses, nepoviruses, sobemoviruses, enamoviruses, bymoviruses, rymoviruses, and some luteoviruses (105, 223). It is not known whether the members of these diverse virus groups employ similar strategies and require similar sequence elements for cap-independent initiation of translation. Some evidence exists for the internal initiation of translation on potyvirus RNAs (13, 203); at the same time, data from different laboratories concerning the mechanism of ribosome entry in CPMV RNA are conflicting (19, 352). Construction of a bicistronic expression cassette with a segment of the 5′-UTR from a potyvirus, PVX, has been reported (203).

(ii) Leaky ribosome scanning. Most eukaryotic mRNAs are monocistronic. The eukaryotic 40S ribosome subunit forms a complex with initiation factors and with initiator methionyl-tRNA and is believed to bind mRNA at or close to its 5′ end and then to scan inward until the initiator AUG codon is recognized (187, 322). Efficiency of recognition depends on the sequences flanking the AUG, on the length of the 5′-UTR, and on the secondary structure of the sequences around the AUG; if any of these features are suboptimal in the first AUG, it might be bypassed in a process known as leaky scanning, and a downstream AUG codon will be preferentially recognized (187). An A residue at −3 and a G residue at +4 are considered to form the optimal context in plants (50).

In tymoviruses, two ORFs start near the 5′ end of genomic RNA, overlapping in different coding phases. A similar arrangement is observed in luteovirus ORFs 3 and 4 which are expressed from the same subgenomic RNA (Fig. 5). In these cases, both ORFs are translated in vitro and in vivo; mutation of each AUG eliminates translation of its respective ORF (35, 223, 358). The upstream AUG is in each case flanked by suboptimal residues, whereas the context of the downstream initiator is optimal. It is likely that leaky scanning is operating in these cases. Additionally, in BYDV-PAV, the sequences close to the downstream AUG influence the efficiency of initiation at the upstream AUG, presumably by melting a base-paired region that limits access to the upstream AUG (74, 223).

Leaky scanning is also a plausible explanation for the simultaneous expression of the nested genes from the polyfunctional subgenomic RNAs in tombusviruses (280) and enamovirus (68).

Three ORFs are thought to be expressed from the pregenomic polycistronic RNA in a group of plant pararetroviruses, badnaviruses (285). The rules of choice of the optimal AUG codon are exceptional in this case; i.e., initiation of the smaller upstream ORFs occurs at non-AUG triplets, albeit at low efficiency. The AUG codon of the major ORF in these genomes is the first initiator from the 5′ end that has the optimal context (285).

(iii) Translational frameshift. In several groups of plant viruses, expression of the 5′-proximal cistron on the genomic RNA results in synthesis of a relatively short protein in a predominant amount. However, a significant portion of the ribosomes engaged in translation undergo a shift in the translational reading frame. This results in the synthesis of a reduced amount of a larger protein. In luteoviruses, enamovirus, and dianthoviruses, a −1 shift in the reading frame occurs (68, 179), whereas in closteroviruses, a +1 frameshift is observed (4, 175); in all cases, the larger protein is the putative replicase.

Frameshifts have been commonly observed in animal and yeast viruses (36, 78, 163), as well as in some bacterial genes and in protists (11, 99, 123, 147). Frameshifting is one example of the so-called recoding of RNA, whereby the linear readout of triplets, or meaning of the triplets, is altered at particular sites within the mRNA (11, 123).

Mutational analysis of the frameshift sites in plant virus genomes and in other genes indicates that these sites share several conserved features. The actual slippage of a ribosome that changes reading phase occurs at a "shifty" heptanucleotide in which three identical residues (As, Gs, or Us) are followed by one of the four tetranucleotide stretches, UUUA, UUUU, AAAC, or AAAU (162, 223). The rate of frameshifting at this site is, however, negligible in the absence of an additional signal, a *cis* element with a particular secondary structure in the vicinity of the shifty heptanucleotide (162, 223, 266). Most commonly, this RNA element is located downstream of the slippery site and can potentially form either a stable stem-loop or a pseudoknot, or sometimes both (188, 266, 331). When the frameshift signals of a luteovirus, BYDV-PAV, were analyzed in a wheat germ translation system, additional sequences, located far downstream in the virus genome but distinct from the 3′ sequences required for cap-independent initiation of translation, were found to be required (223) (Fig. 5).

The position of the termination codon for the upstream ORF appears to be of minor importance for frameshift events; in beet yellows closterovirus, the +1 frameshift site is immediately upstream of the 1a protein termination codon (4), whereas in the related citrus tristeza closterovirus, the identical frameshift site is separated from the nearest termination codon by several dozen nucleotides (175).

Recently, a simplified version of a −1 frameshift signal from BYDV-PAV, lacking the far-downstream activator elements, was shown to ensure expression of a frameshift product in *E. coli* at a calculated rate of 3% (73). Thus, construction of bicistronic frameshift-mediated expression modules for plants seems to be a realistic expectation.

(iv) Leaky termination. In another example of RNA recoding, stop codons of many viral (and some nonviral) genes are occasionally read by ribosomes as sense codons, presumably as a result of recognition of these codons by rare isoacceptor tRNAs (11, 123, 202, 223). This results in production of a pair of N-coterminal proteins, with the readthrough product being less abundant. In plant viruses, a leaky termination codon was first discovered in TMV (252), and similar strategies have been since described in tobamoviruses, tobraviruses, furoviruses, tombusviruses, carmoviruses, luteoviruses, and machloviruses (223, 314, 315). It is thought that the occurrence of a host-encoded suppressor tRNA is the major factor in translational readthrough (17, 202, 223, 373); however, *cis* elements that contribute to the overall efficiency of the process have also been characterized for some animal viruses, e.g., a downstream pseudoknot in the case of Moloney murine leukemia virus (365).

A detailed mutational analysis of a leaky termination codon in TMV has been performed (314, 315). This codon occurs within the replication protein of this virus (Fig. 4). The readthrough product, the 183-kDa protein, contains methyltransferase, helicase, and polymerase domains; the shorter, 123-kDa protein, lacks polymerase. The relative amount of 123- and 183-kDa proteins is close to 30:1 in vivo (127, 252). Both polypeptide species are essential for replication (65). Mutagenesis experiments revealed that the *cis* sequence required for readthrough is in this case extremely compact, the major read-

through signal residing in only two codons immediately downstream of the leaky UAG terminator. The sequence of the codon preceding UAG also modulates readthrough efficiency (314, 315).

Recently, this readthrough signal was inserted into a novel position in a TMV expression vector just downstream of the capsid protein cistron to achieve the expected amount of a readthrough product in vivo (143; see the section on peptide display on the surface of plant virus particles, below).

(v) Polycistronic translation in caulimoviruses. Leaky scanning, frameshifting, and leaky termination all result in the expression of more than one polypeptide from a single stretch of eukaryotic mRNA; the products are in each case coterminal or overlapping. Inclusion of multiple IRES elements on an mRNA results in translation of nonoverlapping polypeptides but requires several nonidentical IRES elements to reduce recombination (369).

A distinct mode of translational control is used by caulimoviruses. Their genome-length, terminally redundant RNA transcript is multifunctional. It is the pregenomic RNA that is reverse transcribed to produce virion DNA (156, 285). The full-length RNA intermediate may serve as the form in which the virus genome is transported from cell to cell (55, 276). It is also a polycistronic mRNA for expression of several virus genes (31, 137, 302).

Upon CaMV infection, only two major virus transcripts are found in vivo. The 35S pregenomic RNA spans the whole genome, while the 19S subgenomic RNA is the messenger for the synthesis of a single polypeptide, the product of CaMV gene VI (Fig. 1). Thus, multiple ORFs (five to seven in different caulimoviruses) have to be expressed from the full-length RNA, the task being further complicated by the occurrence of several AUG codons and the extensive secondary structure in the long leader of the transcript (156, 285).

No IRES-like elements or recoding signals have been found in the caulimovirus full-length transcript. Instead, it has been determined that polycistronic translation from the pregenomic RNA is mediated in *trans* by the protein product of the independently expressed ORF VI (31, 111, 137, 302). When CaMV and FMV derivatives with a reporter gene inserted in frame with a downstream ORF are electroporated in plant protoplasts, they are expressed only in the presence of an ORF VI product provided in *trans* (137, 302). Interestingly, the ORF VI protein of CaMV and of other caulimoviruses activated the expression of FMV-derived constructs (94, 137). Moreover, it has been shown that bicistronic reporter constructs, virtually free of any virus sequences, expressed both reporter genes in the presence of CaMV ORF VI protein (111).

Deletion analysis of CaMV ORF VI has shown that an N-terminal fragment of ca. 120 amino acids retains substantial translation activation capacity (70, 71). It has been observed that this segment contains the most highly conserved block in the otherwise divergent sequences of ORF VI proteins (71).

To be efficiently expressed in the presence of the *trans*-activator protein, ORFs on a polycistronic RNA must be closely spaced. Several nucleotide overlaps between adjacent genes or small insertions between an upstream termination codon and a downstream AUG are frequently observed in caulimovirus genomes and support efficient polycistronic translation (233, 285).

The mechanism of ORF VI-mediated translation of downstream cistrons from the polycistronic mRNAs is unknown. Binding of ORF VI products of CaMV and FMV to RNA in vitro is weak and nonspecific, whereas the 120-amino-acid mini-*trans*-activator does not bind to RNA at all (70, 94); on the other hand, the product of ORF VI has been found in complexes with polysomes (285).

In an attempt to achieve translationally inducible gene expression in planta, a portion of the CaMV genome consisting of gene VII and the initiator codon of gene I was fused to the GUS reporter gene (375). This bicistronic module was inserted between the CaMV 35S promoter and the CaMV polyadenylation signal in a plant transformation vector, and transgenic *Arabidopsis* plants were obtained. GUS activity was extremely low in these plants, as would be expected for an expression product of a gene that is 5′ distal on a conventional eukaryotic mRNA. In contrast, when transgenic plants were infected by CaMV or when the ORF VI product was introduced by a cross with an *Arabidopsis* line transformed with the ORF VI gene, strong expression of the GUS activity was observed (375). Similar results were obtained with another caulimovirus, peanut chlorotic streak virus, in which GUS reporter expression was shut down after fusion of the reporter to an internal cistron but was released in the presence of the replicating virus (211).

In principle, any closely spaced array of several genes positioned head to tail with their own initiator and terminator codons downstream of the 5′-proximal silencing ORF might be inducible by caulimovirus infection or by ORF VI protein. However, efficient translation of polycistronic RNA may require auxiliary 5′ and 3′ *cis* elements, as demonstrated for FMV (94, 136, 304).

Posttranslational Protein Modification and Sorting

Dozens of plant virus genomes, representing many virus groups, have been fully or partially sequenced. Computer analysis of amino acid sequences of plant virus proteins has often allowed one to predict their properties (185, 232). However, little is known about the intercellular sites where plant virus proteins are synthesized or about the pathways of protein processing and sorting in vivo. In this unexplored area, the potyvirus group with its best-studied representative, TEV, makes a notable exception.

Potyviruses express their ca. 10-kb genomes via synthesis of a single precursor polyprotein that is cleaved into mature virus proteins by the concerted action of three virus-encoded proteases (88, 351) (Fig. 6). The specificity and properties of these proteases, especially of the NIa protease, have been extensively studied. The unique specificity of NIa protein has turned it into a useful tool for many applications, some of which are considered below.

In potyviruses and in related bymoviruses, some viral proteins are transported to the nucleus of infected cells. Although the biological significance of their nuclear localization is uncertain, these proteins have been used as a model system to investigate the principles of nuclear import of proteins in plant cells (45, 272, 273).

An interesting example of intercellular sorting and assembly, apparently relevant to biological function, is demonstrated by plant virus movement proteins. This group of structurally diverse proteins mediates cell-to-cell transport of plant virus genomes via plasmodesmata (69, 210, 232). Movement proteins from several virus groups are able to specifically interact with plasmodesmata and to modulate their permeability.

Potyvirus NIa proteinase and the controlled processing of proteins. The genome of TEV is a positive-strand RNA of ca. 9.5 kb that has a protein (VPg) attached to its 5′ end and a poly(A) tail at its 3′ end. Upon genome expression, a single polyprotein precursor of 351 kDa is synthesized. Three virus proteases catalyze cleavage of the 351-kDa polyprotein into

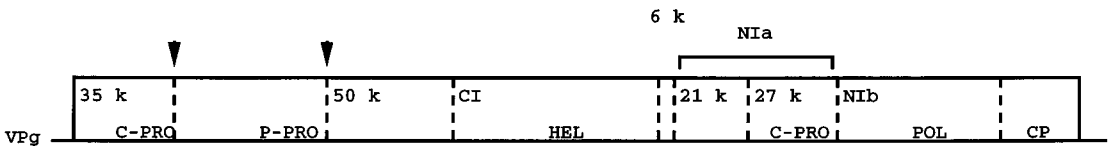

FIG. 6. Organization of the genome of TEV (potyvirus group). The 9.5-kb genomic RNA of TEV is indicated by the horizontal line. The virus polyprotein is shown by the open box. The sites of the polyprotein cleavage are indicated by the broken lines. Arrowheads indicate the sites at which 35-kDa and HC-PRO proteases cleave their respective C termini; other cleavages are performed by the NIa protease. VPg, covalently linked protein at the 5′ terminus of virus RNA (part of the NIb protein); C-PRO, chymotrypsin-like protease; P-PRO, papain-like protease; HEL, helicase; POL, putative RNA-dependent RNA polymerase; CP, capsid protein.

intermediates and then into final products; these proteases are P1 protease (serine type), HC-Pro (a papain-like protease), and NIa (a serine protease) (43, 46, 87, 245, 351). P1 and HC-Pro are responsible for proteolytic release of their respective C termini, whereas NIa cleaves at six major (and probably at a few cryptic) sites within the rest of the polyprotein (Fig. 6).

Extensive studies of cleavage events mediated by the NIa protease expressed from different cloned portions of the TEV genome, as well as site-directed mutagenesis based on computer-aided comparisons of NIa with other proteases, have provided insights into the properties of this enzyme. Some features of NIa appear to be shared with the proteases of the related comoviruses and animal picornaviruses, and others are unique (88). The NIa protein is autocatalytically released from the 351-kDa precursor predominantly as a 49-kDa polypeptide, although the 55-kDa intermediate also is found. In this form, NIa is deposited in the nucleus (hence its name, for nuclear inclusion protein a [nuclear inclusion protein b also exists] [Fig. 6]). The 49-kDa protein is additionally autoprocessed into the N-terminal 21-kDa protein and the C-terminal 27-kDa protein that retains proteolytic activity and is the final form of the NIa protease (88). As in other virus proteases related to cellular serine proteases, the catalytic Ser residue in NIa is replaced with Cys. NIa cleaves polypeptides both in *cis* and in *trans* at a specific heptapeptide cleavage sequence determined as EX[IVL]YXQ-[SG] (X indicates any amino acid residue, the letters in brackets indicate alternative residues, and the hyphen indicates the cleavage site [88]). Variable residues in this signal influence the extent of its cleavage.

The high specificity of NIa protease makes it useful in a variety of applications. For example, a system for affinity purification of overexpressed fusion proteins is commercially available. The affinity tag is linked to a protein of interest via the heptapeptide sequence, which is recognized and specifically cleaved by NIa (7).

NIa protease might be used to express multiple proteins from a single transcriptional unit. In a recent study, coat protein genes of several plant viruses were engineered into a single ORF, in which several heptapeptide cleavage sequences were inserted. The NIa gene itself was also fused to the same ORF. Upon transcription and translation in vitro, individual coat proteins were efficiently released (212). When expressed as a single gene in transgenic plants, this construct may confer protection against several viruses.

In a recent work, NIa and its target sequence were used for the development of a system for selection of proteases with given specificity in yeasts (321). A DNA fragment coding for the NIa cleavage sequence was inserted between two domains in a yeast transcriptional activator gene; the product of this fusion remained active, inducing pathways of metabolism of a suicide substrate. The yeast cells could grow only when transformed with the NIa gene, because the protease then cleaved the transcriptional activator at its cognate cleavage site. This system is used to screen for proteases and protease inhibitors (180).

NIa homologs of other potyviruses have distinct cleavage sites that might be as highly specific as in the case of TEV (88).

Nuclear localization signals in potyvirus proteins. Two proteins of TEV, the NIa protease and the 58-kDa NIb protein (putative RNA polymerase), are found in the nuclei of virus-infected tobacco plant cells (204, 272). In some other potyviruses, one or both of the equivalent proteins may also be transported to the nucleus (305). It is not known whether the nuclear location of these proteins is essential for the virus infection or whether the nuclear localization signal (NLS) in potyvirus proteins is fortuitous. Nevertheless, dissection of NLS in TEV proteins has been performed (45, 204, 272, 273).

Two types of NLS are generally recognized in eukaryotic proteins. The first type, specified by the NLS from the large T-antigen of an animal papovavirus, simian virus 40, consists of a contiguous stretch of four or more positively charged (lysine and arginine) residues. The second type, specified by the NLS of a nuclear chaperone protein, nucleoplasmin, consists of two amino acid stretches rich in positive residues, separated by several to several dozen amino acids (75–77). Clusters of positively charged residues have been noticed in both NIa and NIb proteins of TEV (272). Cloned NIa and NIb cistrons or their derivatives with various point mutations and deletions were fused to the GUS reporter gene, and localization of chimeric proteins was investigated in transient-expression assays and in transgenic plants expressing the corresponding genes.

In the case of NIb protein, mutations in the putative NLS sequences abolished nuclear transport; however, a similar effect was achieved by alterations made in other regions, especially when these alterations were likely to change protein tertiary structure (204). Thus, NLS in the NIb protein is quite complex, probably including both amino acid sequence elements and interactions of higher order within the molecule (204).

In contrast, a distinct NLS was discovered close to the N end of the 49-kDa NIa protein (45). This bipartite NLS consists of two additively acting domains, one between residues 1 and 11 and another between residues 43 and 72. Each domain is lysine and arginine rich. The ca. 250-nt segment of the TEV genome encoding this NLS can be fused in frame to either the 5′ or 3′ ends of heterologous genes to direct their efficient nuclear uptake in vivo (45).

Recently, nuclear localization in vivo and the occurrence of the bipartite NLS have been described for the coat protein of barley yellow mosaic virus, a bipartite RNA virus related to potyviruses (271).

A series of expression cassettes that contain the GUS reporter gene flanked by segments derived from TEV and then by two more segments of CaMV were constructed by Carrington and colleagues (45, 272). CaMV sequences are the 35S promoter with double enhancer and the polyadenylation signal. The 5′ untranslated region of TEV enhances translation of the GUS gene. Upon transformation with or transient expression of this plasmid, GUS activity is found in the cytoplasm. In some derivatives of this construct, the sequence coding for the

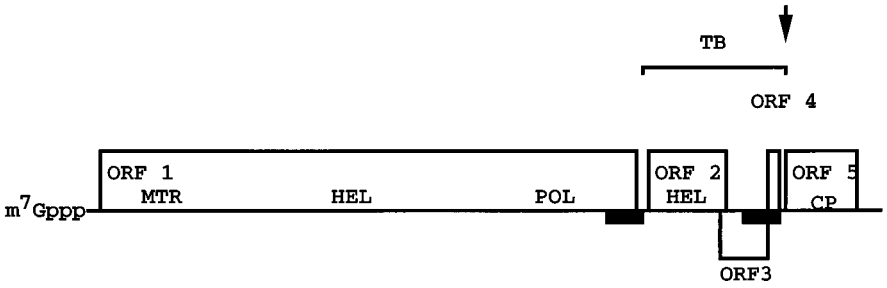

FIG. 7. Organization of the genome of PVX (potexvirus group). The 6.5-kb genomic RNA of PVX is shown by the horizontal line. Individual ORFs (ORFs 1 to 5) are shown by open boxes. Black rectangles indicate positions of two subgenomic promoters on the complementary RNA strand. The vertical arrow indicates the site where foreign genes followed by the second copy of the subgenomic promoter are inserted into the PVX-derived expression vector. m^7Gppp, cap structure at the 5′ terminus of genomic RNA; MTR, putative methyltransferase domain; HEL, putative helicase domains; TB, triple-gene block of cell-to-cell movement proteins; CP, capsid protein.

NLS of the NIa protease is inserted downstream of the GUS gene; expression of this plasmid results in localization of GUS activity in the nuclei. All modules in this cassette are separated by the unique restriction sites. Such a construct can be used to test new promoters, reporters, enhancers and sorting signals (1, 45, 272).

Cell wall retention of plant virus movement proteins. Plant virus movement proteins (MPs) facilitate cell-to-cell spread of virus infection. By mutational analysis and by sequence comparisons with the known MPs, movement function has been assigned to proteins encoded by viruses from more than 20 groups (69, 232).

The largest class of MPs includes proteins encoded by at least 15 groups of viruses representing all major genome strategies (232). This class is characterized by a shared amino acid motif and has been designated the 30-kDa superfamily, after the best-studied MP of tobamoviruses. Upon virus infection or when expressed in transgenic plants, members of this superfamily of proteins are found in the cell wall fraction and, more specifically, in plasmodesmata. Some members of the same superfamily form tubular structures protruding from plasmodesmata. MPs of caulimoviruses and comoviruses were found both in cell walls and within tubules (206, 216, 349), indicating that the two types of locations might represent different aspects of a complex picture. Members of the 30-kDa superfamily from tobamovirus, cucumovirus, dianthovirus, and geminivirus groups were shown to functionally modify plasmodesmata, increasing their size exclusion limit (69, 74a, 110, 240, 354), i.e., making plasmodesmata penetrable for macromolecules, including MPs themselves.

How MPs enable virus translocation through plasmodesmata, or how these proteins are sorted inside the cell, is not known in detail. Deletion analysis has shown that certain C-terminal truncations of tobamovirus MP or N-terminal truncations of alfamovirus MP prevent their cell wall localization as well as virus cell-to-cell spread (24, 97, 112). In neither of these cases was it determined whether the removed domain was indeed a cell wall-targeting signal or whether it disrupted the structure of such a domain located elsewhere in the protein.

Another group of MPs is the so-called triple-gene block found in several groups of positive-strand RNA viruses. Two of the three genes in this block are predicted to be membrane proteins; the third, a putative helicase, is related to the replicative helicases of the same viruses (185, 232) (Fig. 7).

Obviously, the possibility of targeting proteins to plasmalemma or to plasmodesmata in vivo would be of use in plant cell biology. Plant virus MPs might be a source of appropriate targeting signals. One can speculate that in the proteins from the 30-kDa superfamily, the conserved 30-kDa motif (232) is part of such signal.

Recently, a domain in the 30-kDa MP of TMV that is sufficient for increasing the plasmodesmatal size exclusion limit was mapped (354). Expression of this domain in plants is expected to be a powerful tool to explore cell-cell communications, metabolite flow, and other aspects of plant "supracellular" organization (210).

Encapsidation of Tagged RNAs—Coat Proteins and Origin-of-Assembly Signals

The discovery, in the 1950s, of self-assembly of infectious virions from purified genomic RNA and capsid protein of TMV (104) was one of the most important contributions of plant viruses to the emergence of molecular biology. The structural basis of this remarkable example of specific protein-RNA interactions has been well studied since then (238, 340).

A discrete 75-nt sequence of TMV genomic RNA, at nt 5444 to 5518 from the 5′ end (127), is sufficient to initiate spontaneous assembly of rod-shaped virions in the presence of virus coat protein in vitro (340). Two important features of this origin-of-assembly sequence (OAS) are the occurrence of G residues at every third position and a 14-bp stem with a 9-nt loop formed within this segment. The OAS interacts with oligomeric capsid protein aggregates to form a nucleation complex (48, 246). Assembly then proceeds toward both ends of the RNA molecule by stepwise encapsidation (48, 208, 341). The elongation step is sequence independent; any RNA that contains the OAS will be encapsidated, regardless of its length (367). The 5′ direction of assembly seems to be more efficient than the 3′ direction. Hence, the encapsidation process is completed sooner when the OAS is positioned closer to the 3′ end of the RNA (208, 246, 367).

The 6.4-kb genome of TMV is completely encapsidated in vitro within 6 min (208). The resulting ribonucleoprotein (virion) of TMV is remarkably stable. TMV retains infectivity in processed tobacco products indefinitely, and preparations of the virus virions obtained in the 1930s, when stored under refrigeration, are infectious and contain mostly undegraded RNA (305).

It has been proposed that upon penetration into plant cells, TMV particles are uncoated cotranslationally (306, 366, 367). In vitro, eukaryotic ribosomes are able to translate the 5′-proximal 126-kDa cistron from encapsidated TMV RNA after brief treatment of virions at pH 8 (306, 366). However, prokaryotic ribosomes will uncoat and translate RNA from intact, untreated virions (367). A lack of G residues in the 5′ leader of

TMV RNA might account for weaker interactions of capsid protein with this RNA segment (318, 367).

On the basis of these observations, strategies for tagging of foreign genes with TMV encapsidation signals were developed (316, 317). Chloramphenicol acetyltransferase (CAT) and GUS reporter genes were fused to cDNA copies of the OAS; the RNAs obtained by in vitro transcription of these constructs were efficiently encapsidated by TMV coat protein both in vitro and in transgenic plants. The resulting TMV-like rodlets were stable and RNase resistant, and encapsidated genes could be expressed in vitro and in vivo (316). In an even more versatile version of this approach, the TMV coat protein and a tagged transcript were coexpressed in *E. coli* and gave high yields of the encapsidated RNA (160).

A chimeric RNA based on the genome of BMV, a virus with isometric virions, was engineered to encapsidate as a virion with helical symmetry (293). In RNA 3, the BMV capsid protein cistron was replaced by its counterpart from the cowpea strain of TMV. In this strain, the functional OAS resides in the capsid protein gene itself. RNA 3 of this chimeric virus was replicated in protoplasts in the presence of BMV RNAs 1 and 2, subgenomic RNA 4 was transcribed and translated, and TMV-like rodlets were formed in vivo after encapsidation of the OAS-containing RNAs 3 and 4 by TMV capsid protein (293).

Induction of Pathological Effects

Plant viruses induce a variety of symptoms. Signs of infection on aerial parts of plants include abnormalities of pigmentation, abnormalities of growth, and induction of cell death. It is recognized that dramatic differences in symptom appearance can be caused by only a few mutations or a single point mutation in any virus gene or in a regulatory sequence that does not code for a protein (15, 58, 59, 129, 249, 250, 311a, 339). Availability of discrete genetic elements which, when expressed, could induce a specific developmental change in a plant cell would be useful for studies of many aspects of cell biology. One better-studied example of the distinct physiological reaction elicited by a virus protein is the induction of the hypersensitive response (HR).

HR is manifested through the development of a necrotic lesion at the site of virus infection, which restricts the virus from invading other parts of the plant. In *Nicotiana sylvestris*, HR in response to TMV is determined by a single dominant gene, *N′* (65). Many strains of TMV, but not the common strain U1, elicit HR on *N. sylvestris*. By construction of TMV derivatives expressing capsid proteins of different strains or no capsid protein at all, it has been determined that the coat protein is the only virus-encoded component required for induction of *N′*-triggered HR (59). The HR-eliciting signal within the TMV capsid protein is thought to be composed of several amino acids scattered along the length of the protein but juxtapositioned by protein folding (58). A repertoire of elicitor and nonelicitor proteins has been created by mutagenesis (58, 59), and it has been noticed that mutations in the HR-eliciting coat proteins are located at the interfaces between the adjoining subunits in the virion (61), suggesting that HR is triggered by domains in the coat protein which are not exposed upon the wild-type, noneliciting TMV infection (61).

In another example of a toxic protein of a plant virus, a small hydrophobic protein encoded by RNA 3 of beet necrotic yellow vein virus has been shown to induce cell death when expressed in vivo (170). It would be of interest to evaluate the potential of such genes as negative selection markers for whole plants or as inducers in studies of developmental processes like programmed cell death.

Expression of a virus can also be used as a genetic marker. In one case, the CaMV genome was used to monitor intrachromosomal DNA recombination events (113). Virus DNA was extensively rearranged before being cloned into a binary vector and transforming rapeseed plants. Only homologous DNA recombination at a repeated segment of integrated CaMV genome could release the replicating virus. Such events were indeed observed as virus symptoms at low frequency.

PLANT VIRUS GENOMES AS EPICHROMOSOMAL EXPRESSION VECTORS

Viruses That Are Candidates for Vectors

The potential of viruses as vehicles for delivery and expression of foreign genes in vivo is established. Replicating derivatives of bacteriophages and animal viruses are indispensable in a variety of gene transfer applications and are increasingly used to evaluate gene functions or regulation and to produce proteins.

Plant viruses were suggested as possible candidates for gene delivery into plant cells more than a decade ago (see, e.g., reference 311). The advent of efficient *Agrobacterium*-mediated plant transformation, as well as some problems encountered with the simple vectors based on CaMV, a virus with virion DNA thought to be a good choice for a gene vector, switched the attention of researchers elsewhere. In later years, however, plant virus vectors have returned to the spotlight. The revival of interest in virus-based vectors arises from extensive studies of plant virus genome organization, expression, and variation, as well as the technical advances that now enable manipulation of cloned DNA copies of viral RNA genomes (34).

Both the advantages and shortcomings of expression systems based on plant virus replicons are widely recognized (see, e.g., references 57, 84, 157, 230, and 318). Advantages include high copy number of replicating virus genomes per cell, resulting in potentially high expression of an introduced gene; ease of introduction and autonomous spread in plants; quick recovery; and lack of genomic positional effects—all features not available with stably integrated genes. Major disadvantages of virus vectors are their low genetic stability and their pathogenic impact, both of which are especially undesirable in agricultural settings.

Plant virus vectors based on viruses from at least eight groups that employ different genome strategies, e.g., DNA viruses from the geminivirus group, caulimoviruses that have virion DNA but replicate through an RNA intermediate, and positive-strand RNA viruses, have been now constructed.

Virus-based vectors can be created either by deletion of portions of the genome with substitution of foreign sequences in their place (replacement vectors) or by addition of a foreign gene without removal of any part of the virus genome (insertion vectors). Plant virus-based replacement vectors may be defective and require helper functions provided by a fully competent virus or by a transgenic plant, or they can replicate and accumulate in vivo autonomously. Depending on the type of the construct, vectors might require a different means of delivery into plants, most commonly mechanical inoculation or agroinfection.

Recent advances in the development of plant virus vectors seem to provide new insights into several aspects of virus variation and evolution, as related to epichromosomal replicon stability in plants. The high spontaneous mutation rate in viruses was for a time a matter of speculation; recent results (178) tend to refute that notion, suggesting that mutation rates

of plant RNA viruses might be much lower than had been believed.

Bromoviruses and hordeiviruses. Bromoviruses and hordeiviruses are two groups of positive-strand RNA viruses with tripartite genomes. The genomic RNAs of bromoviruses and hordeiviruses are capped and possess tRNA-like structures at their 3′ termini. Both groups encode related replicative proteins with at least three functional domains. In bromoviruses, replicative proteins are encoded by RNAs 1 and 2 (Fig. 3). In hordeiviruses, the equivalents of these proteins are encoded by RNAs α and γ. In both groups, capsid protein and MP(s) are encoded by the remaining RNA and its subgenomic derivative. Both groups include viruses that infect cereals, a family of plants which are difficult to transform and for which virus vectors are actively sought. Both bromovirus- and hordeivirus-derived autonomous vectors have been evaluated only in protoplast systems thus far (108, 168).

The capsid protein of a hordeivirus, barley stripe mosaic virus, is encoded by the 5′-terminal cistron on the RNA β segment; the triple-gene block of movement proteins is 5′ distal and is thought to be expressed via a subgenomic RNA (164). The capsid protein is dispensable for virus RNA replication, for cell-to-cell movement, and, at least in some hosts, for long-distance movement (164). In bromoviruses, the 32-kDa MP is 5′ proximal on RNA 3 whereas the capsid protein is expressed from the subgenomic RNA arising from RNA 3. In this group, both MP and capsid protein are required for cell-to-cell movement but are dispensable for RNA replication (6).

A bacterial CAT reporter gene was used to characterize expression of a foreign gene from a bromovirus vector. The CAT gene was inserted in frame with the BMV capsid protein initiation codon, while the remaining, although nonfunctional, part of the capsid protein gene was either retained downstream of the CAT gene or deleted (5, 108). RNAs were obtained by in vitro transcription, capped, and inoculated to barley protoplasts. The levels of modified RNA 3 and RNA 4 were lower than for the wild-type virus, probably because of lack of encapsidation. Despite the decrease of RNA replication in the vector compared with the wild type, CAT activity per milligram of cellular protein was several times higher than in the case of expression of the CAT gene in stably transformed transgenic plants (108). As the modified BMV did not express capsid protein and could not move from cell to cell, the expression level of the foreign gene and stability of this vector in whole plants could not be determined.

Recently, a BMV vector was designed to efficiently initiate capsid protein synthesis at an alternative, downstream AUG. The human gamma interferon gene was fused to this downstream initiator, located 24 nt from the initiator for the full-length protein. This arrangement resulted in high expression of the interferon gene in protoplasts, at a level of 5 to 10% of total extracted protein at 24 h postinoculation (228). It is thought that the 24 nt derived from the coding sequence of the full-length capsid protein enhanced foreign gene expression (228).

A hordeivirus vector has been engineered (168). In a cDNA copy of barley stripe mosaic virus RNA β, the firefly luciferase gene was fused in frame to the 5′-terminal portions of either the capsid protein gene or the putative movement-associated helicase gene. RNA transcripts of the mutant constructs were inoculated into tobacco and maize protoplasts together with the wild-type RNAs α and γ. RNA in which the triple-block helicase was replaced could replicate in the presence of RNAs α and γ, and high luciferase activity was observed in these protoplasts. RNA with the capsid protein gene replaced by the luciferase gene was nonamplifiable in protoplasts, and luciferase was expressed only transiently. This was somewhat unexpected, because a functional capsid protein was known to be dispensable for virus replication and movement (164). Apparently, a *cis* element essential for replication was disturbed by the replacement of this gene by the luciferase gene (168).

Tombusviruses. Tombusviruses are a group of positive-strand RNA viruses with icosahedral capsids and a single genomic RNA of about 4,800 nt. The 5′-terminal gene encodes a ca. 33-kDa protein, which is extended to a 90-kDa protein by the readthrough of a leaky termination codon (303). The latter protein is thought to be the RNA replication enzyme (185). The downstream genes are expressed via subgenomic RNAs. At the 3′ terminus is a pair of nested proteins, one of which is the putative cell-to-cell MP (62, 303). In the intermediate location is the capsid protein, which is dispensable for replication and systemic movement (62, 280, 303). A notable feature of tombusviruses is that they produce large amounts of defective interfering (DI) RNAs in vivo; these RNAs are mosaics of terminal and internal portions of the virus genomic RNA (154) and are rapidly generated de novo in plants infected with full-length RNA transcripts of cloned virus DNA (40). Presumably, the DI particles in tombusviruses and other RNA viruses result from errors intrinsic to the copy choice mechanism of RNA replication.

The tomato bushy stunt tombusvirus genome was engineered to express reporter genes fused in frame to the beginning of the capsid gene or to either of the two nested 3′-proximal proteins (303) (Fig. 1). As expected, mutants with the replaced movement gene did not move from cell to cell, although they replicated in protoplasts and efficiently expressed the reporter gene. A mutant in which the GUS gene replaced most of the capsid protein cistron could replicate at a reduced level in protoplasts and in *Nicotiana benthamiana* plants after mechanical inoculation with RNA transcripts. Substantial GUS activity was observed in the inoculated leaves, permitting histochemical visualization of virus spread (303). Virus symptoms, somewhat milder than in the wild-type infection, were also observed in the noninoculated leaves. Reporter gene expression was quite low in these leaves, and it was shown that the majority of virus progeny did not retain the added gene, presumably having deleted it by a mechanism similar to that of DI RNA formation (303).

In a complementary approach, cymbidium ringspot tombusvirus, closely related to tomato bushy stunt virus, was used as a helper which is able to replicate its own DI RNAs (41). In this case, a cDNA copy of one naturally occurring DI RNA species was engineered to express capsid proteins from tomato bushy stunt virus, or from the unrelated tomato aspermy cucumovirus, under the control of the leader and the ATG codons present in this DI RNA. Two different sites within DI RNA of cymbidium ringspot virus were used for the insertion of a foreign gene. Modified DI RNA transcripts replicated in vivo in the presence of the replicating helper cymbidium ringspot virus and attenuated the symptoms due to the latter (41). The stability of the insert varied with the site of insertion. When the insertion was stable in planta, the expression level of the coat protein from the DI RNA was only a few-fold lower than that with the infection of the wild-type virus from which it was derived (41).

Potexviruses. Potexviruses are positive-strand RNA viruses with monopartite genomes and filamentous virions. They express a replication-associated protein from their genomic RNA and also synthesize subgenomic RNAs for the expression of the triple block of movement proteins and the capsid protein (16, 51, 228a) (Fig. 7).

A potexvirus, PVX, has been engineered to express a GUS reporter gene (51). In one construct, most of the capsid protein gene was removed from the full-length cDNA copy of PVX and a derivative of the GUS gene was fused in frame to the remaining five 5′-terminal codons of this gene. It was shown that the transcripts of this construct replicated poorly in protoplasts, in contrast to the high infectivity of wild-type transcripts (51). Histochemical detection of GUS activity in situ revealed that replication of the vector was confined to the initial sites of infection (51). Apparently, the coat protein gene sequence or its expression product is required for both efficient RNA replication and cell-to-cell spread of PVX.

To maintain all vital functions in the PVX-based vector, another construct, in which a copy of capsid protein gene and its own subgenomic promoter was inserted downstream of the GUS gene, was engineered. This construct thus contains two copies of the subgenomic promoter for the capsid protein, one directing synthesis of a wild-type mRNA for the capsid protein itself and the other enabling transcription of an additional subgenomic RNA that would express the GUS gene (51) (Fig. 7). This vector replicated in protoplasts and in inoculated plants. GUS activity was detected throughout the plant. Upon systemic spread, however, partial loss of the foreign gene was observed in the progeny (51). Loss of the entire GUS gene by spontaneous deletion of RNA was most common, presumably because repetition of identical subgenomic promoter sequences at the 5′ and at 3′ ends of the GUS gene contributed to homologous RNA recombination. Occasionally, RNA species of intermediate size were also observed, indicating that nonhomologous recombination events had taken place as well (51). Nevertheless, the vector based on PVX has recently been used to rapidly assay the in vivo activity of the cellular enzyme protein kinase Fen and its mutated derivatives (283a).

Potyviruses. The potyvirus group is the largest known group of plant viruses. Many potyvirus diseases are of the utmost economic importance and are common in both dicot and monocot hosts. Potyviruses belong to the picornavirus-like lineage of positive-strand RNA viruses (128, 185) and express their ca. 10-kb genome via a single polyprotein that is cleaved cotranslationally and posttranslationally by three virus-specific proteases, P1, HC-Pro, and NIa (Fig. 6). The N-terminal portion of the polyfunctional HC-Pro protease is required for aphid transmission of virus particles but is dispensable for mechanical transmission (82). The N-terminal portion of NIa protein contains the VPg, which might serve as a primer for virus RNA replication (231). Two proteins, CI and NIb, are virus replication proteins, the helicase and RNA-dependent RNA polymerase, respectively (185, 191, 192). The capsid protein of a potyvirus, TEV, is involved in virus cell-to-cell and long-distance movement, aphid transmission, and some aspect of RNA replication; many of these functions can be mutated separately (80). Potyviruses do not appear to produce subgenomic RNAs or DI RNAs.

Two strategies seem plausible for the development of potyvirus vectors. Because initiation of translation of potyvirus RNA occurs independently of the 5′ terminus, an IRES element might be added to the virus genome downstream of virus genes. As discussed above (see the section on internal ribosome entry), a potyvirus *cis* element with the properties of an IRES has been characterized in transient-expression assays (203).

A foreign gene can also be inserted into a nonessential portion of the potyvirus genome, in frame with the virus polyprotein, to avoid negative effects on downstream gene expression. This strategy has been successfully employed to fuse a GUS gene to the HC half of the HC-Pro gene (82) (Fig. 6). A site for efficient processing by the NIa protease was engineered at the C terminus of the GUS protein. It has been shown that after inoculation of tobacco plants by chimeric RNA transcripts, the virus replicated, released functional GUS protein, and moved locally and systemically, although at a lower rate than the wild type and without causing typical symptoms (82). A high level of GUS activity was observed in the inoculated leaves and other parts of the infected plants, enabling careful analysis of virus association with different cell types (79, 80, 82, 205).

The TEV vector appeared to stably express the added GUS gene upon numerous passages from plant to plant, especially when extracts of inoculated leaves were used for the transfer. However, upon prolonged passages, the insert was gradually lost by stepwise deletions (81, 82).

Tobamoviruses. Engineering of a gene vehicle from the TMV genome illustrates how developments in virus biology contribute to its practical use as a vector. Conversely, a virus vector can be used to address matters beyond that of protein production in plants.

TMV expresses replication-associated proteins from its genomic RNA and employs two subgenomic RNAs for expression of the 30-kDa MP and the 17.5-kDa capsid protein (Fig. 4). The subgenomic promoter for synthesis of the capsid protein mRNA resides within the coding sequence of the 30-kDa protein gene (65, 200, 201), and the coding sequence of the capsid protein apparently contains signals for minus-strand RNA synthesis (65). The 30-kDa protein is required for cell-to-cell movement of the virus, and lesions in the 30-kDa cistron render the virus unable to produce systemic infection, although such mutants are still able to replicate in protoplasts. The capsid protein is not required for RNA replication and cell-to-cell movement but is necessary for efficient spread of TMV from the infected leaf throughout the plant via the phloem (reviewed in reference 65).

In a first-generation TMV expression vector, an internal portion of the capsid protein gene was replaced by the CAT gene in a cDNA copy of the TMV genome. In vitro transcripts were infectious in tobacco plants, viral RNA progeny was detected in the inoculated leaves, and the expression of the CAT gene was observed at an estimated level of 1 mg/g of tissue (329). As expected, neither virions nor systemic movement of virus could be detected in plants. In addition, the replication level and efficiency of virus cell-to-cell movement were impaired (329).

To retain all virus-specific functions, a TMV-based insertion vector was constructed (66). A portion of the 30-kDa protein gene containing a functional subgenomic promoter was fused to the CAT gene, and the resulting fragment was inserted at two different locations in a cDNA copy of the TMV genome. When the CAT gene was placed downstream of the capsid protein gene, the construct replicated poorly in tobacco. In contrast, efficient replication and virus spread were observed in plants that were infected with a construct in which the CAT gene was inserted between the 30-kDa and capsid protein genes (66) (Fig. 4).

The stability of this engineered replicon was analyzed. In inoculated leaves, the CAT insert was largely retained. An additional subgenomic RNA of the expected size was present, and particles of increased length prevailed in virion preparations. Moreover, various levels of CAT activity were observed (66). Transfer of the virus from the inoculated leaves to healthy plants induced new rounds of infection with detectable levels of CAT gene expression.

Some of the progeny from inoculated leaves lacked the added CAT gene, and it was completely lost from the virus

population upon long-distance movement (66). It was concluded that variants with a deleted CAT gene occurred as a result of homologous RNA recombination facilitated by reiteration of the subgenomic promoter sequence and that these deleted variants had an advantage at some step of virus spread.

To further improve the vector, the downstream copy of the subgenomic promoter and the capsid protein gene were replaced by their counterparts from odontoglossum ringspot virus, a tobamovirus closely related to TMV (84). Although functionally equivalent to TMV sequences, the segment of odontoglossum ringspot virus is substantially divergent from TMV at the nucleotide level (84). Either of two selectable markers, a shorter dihydrofolate reductase gene and a longer neomycin phosphotransferase (NPTII) gene, was inserted into the modified vector. The dihydrofolate reductase gene was very stable during subsequent transfers. Some degree of instability was observed with the NPTII gene, but a substantial portion of the virus population retained this gene both in systemic infection and upon serial plant-to-plant passages.

Improved TMV vector was used to achieve high-level expression of a foreign gene in tobacco (189a). A gene encoding a ribosome-inactivating protein, α-trichosantin, was inserted into the TMV expression vector. Plants inoculated with RNA transcripts yielded recombinant protein at a level of 2% of total soluble protein, reportedly one of the highest levels of foreign protein achieved in plants (189a). Recently, a chimeric TMV vector of this type was used to express an enzyme of the carotenoid biosynthetic pathway in tobacco and in another case to inhibit this same pathway by expression of the antisense RNAs of a phytoene desaturase gene (189).

The in vitro transcription step was eliminated by cloning the whole vector between the T-DNA borders of a binary plasmid vector that can be delivered into plants by agroinfection (343). In inoculated plants, the DNA flanked by the T-DNA borders integrated into plant nuclear DNA and was transcribed from the 35S promoter. To ensure that the nonpolyadenylated, tRNA-like 3′ end of TMV RNA is accurately formed, a self-cleaving ribozyme sequence was incorporated into the same cassette. Once released, the TMV vector propagated itself and spread throughout the plant (343).

Additionally, a TMV vector that expresses NPTII sequence was found to be useful in studies of RNA virus gene expression and evolution (62, 171).

Caulimoviruses. The potential of caulimoviruses as plant expression vectors was investigated shortly after molecular characterization of their virion DNA and demonstration of the infectivity of cloned virus DNA (158, 311). The most extensively studied caulimovirus, CaMV, expresses several genes from a polycistronic RNA. However, long untranslated inserts between adjacent cistrons preclude reinitiation of translation of downstream cistrons (156). Therefore, any added gene should either slightly overlap or be separated from the adjacent genes by only a few nucleotides.

Two of the ORFs in the CaMV genome, ORF VII, with no assigned function, and ORF II, encoding the aphid transmission factor, are not needed for CaMV infection (37, 72). Deletion in ORF II had almost no effect on virus accumulation in plants, so this locus has been chosen as an insertion site in most experiments.

In one experiment, most of the ORF II (470 bp) was deleted and the bacterial dihydrofolate reductase gene (234 bp) was fused to the remaining five codons at the 5′ end and the termination codon at the 3′ end of ORF II (37). Genome-length DNA was released from the cloning plasmid and inoculated onto turnip plants. Virus efficiently replicated, expressed the added gene to an estimated level of 8 mg/g of fresh tissue, and conferred some degree of methotrexate resistance to infected plants (37). In a similar type of experiment, a 204-bp metallothionein gene from Chinese hamsters was inserted in place of CaMV ORF II and shown to be expressed (199).

A CaMV vector was further modified by removing most of the dispensable ORF VII (72). The ORF II was then replaced by a larger gene encoding human alpha D interferon (501 bp). The interferon gene was expressed in turnips infected with CaMV vector but was not active in situ, either against CaMV itself or against superinfection with TYMV. However, extracts from the infected plants displayed high interferon activity against animal viruses in cell culture. The yield of interferon produced in turnips was estimated to be about 2 mg/g of fresh tissue (72).

In neither of these experiments was the stability of an insert in the CaMV replicon directly assayed, although it was noticed that slight modifications in engineered areas could have a drastic effect on the infectivity of the vector (37). In one study, the stability of foreign DNA placed just upstream of ORF VII in the otherwise intact CaMV genome was investigated (254). Most of the inserts were efficiently removed upon the first passage. The sensitivity of this region toward insertions was also observed for another caulimovirus, peanut chlorotic streak virus (233). The addition of 204 bp to the CaMV genome was shown to be stable (254). This might be the upper limit of CaMV virion encapsidation capacity.

Recently, peanut chlorotic streak virus, a caulimovirus with a broad host range and a genome organization slightly different from that of CaMV, was proposed as an attractive virus vector (233). Lengthy deletions in three separate genes of this virus did not abolish virus infectivity, suggesting that at least 1.1 kb of its genome is not required for propagation in plants (231a, 233). The 6-bp insertions between several individual ORFs were shown to be stably propagated upon infection, indicating that foreign genes might be introduced into more than one site in the genome (233).

An alternative strategy to increase the coding capacity of the caulimovirus vector might involve construction of a virus derivative with some essential genes replaced by foreign DNA; these incomplete genomes might be rescued by the wild-type virus. This strategy, however, is very inefficient, because defective genomes of CaMV readily recombine in vivo, restoring infectious genotypes (355). As a way to reduce the frequency of virus recombination, a pair of CaMV mutants with long deletions was constructed; in one genome, the deletion spanned most of the essential gene I and nonessential gene II, while in the other, most of gene II and the essential gene III were deleted (155). Because of the overlap in gene II, homologous recombination was impaired, but the two mutants could replicate together by mutual complementation. However, as yet, expression of a foreign gene in this system has not been reported.

Instability of some inserts seems to be inherent in caulimoviruses, presumably because of the high rate of copy choice recombination and of an essential template switch event upon reverse transcription (345). This disadvantage might be counterbalanced by an unparalleled advantage of caulimoviruses, i.e., by the fact that their genomes cloned in bacteria and purified as a bacterial plasmid are highly infectious upon mechanical inoculation of plants, provided that they have reiterated portions of the DNA genome to allow transcription of the full-length, terminally redundant RNA.

Geminiviruses. Geminivirus replication and gene expression have been reviewed recently (29, 230, 336). A feature of geminivirus biology that makes them attractive is that members of

this group infect plants that are difficult to transform by other methods, e.g., monocots and some dicots for which other virus vectors are not known (such as cotton and other members of the family Malvaceae). Another distinction often pointed out is that geminivirus DNA is replicated by cellular polymerases that are most probably proofreading enzymes, resulting in lower mutation rates in these replicons than in RNA virus- or pararetrovirus-based vectors; however, this difference might be less important than has been thought (see below).

Bipartite geminiviruses do not require the capsid protein gene for replication or for local and systemic spread in planta. Vectors based on replacement of the capsid protein gene by a reporter gene, like NPTII, CAT, or GUS, in the A component of TGMV have been described previously (150, 152). Cloned, partially redundant copies of engineered A-component DNA have to be delivered into plants either by agroinfection or biolistically. Such a vector replicates in protoplasts and locally in leaf discs. When coinoculated with the intact B component or onto a plant expressing the B component from an integrated gene, the vector will move locally and systemically (96, 356). Upon systemic movement, the larger GUS gene insert is less stable than the smaller CAT gene insert (150, 356). Apparently, some size constraints exist for bipartite geminiviruses, which are unrelated to virion encapsidation, because the capsid protein was replaced in these vectors.

Monopartite geminiviruses require encapsidation for cell-to-cell and long-distance movement (33, 196); additionally, cell-to-cell movement requires V1 protein (32). Geminivirus vectors based on the MSV and WDV genomes have been constructed by replacement of V1 and capsid protein genes by reporter genes. These vectors replicate only in protoplasts or in primarily infected cells of whole plants (195, 215, 335, 344). A shuttle WDV vector, pWI-11, in which an NPTII reporter gene and prokaryotic origin of DNA replication replace the virus coat protein has been described (344). This vector replicates and expresses kanamycin resistance both in plant protoplasts and in bacteria. Interestingly, pWI-11 rescued a replication-deficient mutant of WDV by providing the C2-C3 replication protein in *trans* (335). A similar system is described in reference 173. A scheme of cross-complementation can be envisaged, in which pWI-11 provides the replicative function to another WDV derivative that carries a foreign gene in place of its replication proteins but can still express capsid protein for packaging both components. It remains to be determined whether this engineered quasi-bipartite geminivirus will be viable and able to spread in plants; obviously, special effort may be needed to minimize reconstitution of the monopartite virus by recombination.

An insertion vector based on the MSV genome has been constructed (309). A cassette comprising the GUS gene between the 35S promoter and *nos* terminator was inserted into the undeleted genomic clone of MSV at a site in the small intergenic region where short insertions were known to be harmless for virus infectivity (308). The vector expressed GUS activity to high levels but did not move systemically unless the added gene was deleted (309). However, when a different reporter, a bacterial *bar* gene encoding phosphinotricine acetyltransferase, was used, it was shown that virus replicates locally to high copy numbers and confers herbicide resistance to the inoculated leaves (310).

Peptide Display on the Surface of Plant Virus Particles

There is considerable interest in producing highly immunogenic peptides by presentation of epitopes translationally fused to the appropriate carrier proteins. Capsid proteins of simple viruses seem to be ideal carriers for antigen display, because each virion exposes the exterior parts of the capsid protein(s) in dozens to hundreds of copies. Bacteriophage-derived systems are routinely used for antigen display (320, 325a). Engineering of plant virus genomes to serve similar purposes would allow one to produce antigens (or other useful peptides) in large quantities in plants without needing elaborate equipment or materials.

The M component of the two-component RNA genome of comoviruses codes for two capsid proteins. On the basis of the known three-dimensional structure of the comovirus virions (209), a domain in the small capsid protein of CPMV that forms a loop on the surface of virions was identified. DNA sequences coding for major antigenic determinants of three animal viruses were inserted into this position. Care was taken to avoid nucleotide repeats flanking the insert. RNA transcripts derived from engineered virus cDNAs were inoculated into cowpeas (263). The modified virus was infectious, and the insert was maintained upon propagation. Encapsidation was unimpaired, and the added peptide appeared to be properly displayed and highly immunoreactive. At least a 30-amino-acid patch could be displayed without destabilization of virions (263).

In another study, a 12-amino-acid peptide, angiotensin I-converting enzyme inhibitor, a medically important antihypertensive agent, was engineered as a fusion to the C terminus of the TMV capsid protein (143). At the vicinity of the termination codon of the capsid protein, gene changes that would enable some degree of translational readthrough were made (315); downstream of this recoding signal, sequences coding for a trypsin cleavage site and for angiotensin I-converting enzyme inhibitor were inserted. The transcript of this clone was infectious, and predicted amounts of the readthrough form of the capsid protein were observed in infected tobacco (143, 326).

Similar approaches have been used to express malaria plasmodium-derived epitopes on the surface of recombinant TMV; judging from epitope recovery, it was estimated that the vaccines obtained in this way would be cost effective (342).

Genetic Stability of Plant Virus-Based Replicons

Viruses experience rapid evolution. Nucleotide substitutions are frequent in RNA viruses, resulting in a population of variant RNAs that survive as a "quasispecies." Remarkably, the quasispecies status is established rapidly, sometimes over only one or a few passages, even when infection is started by a genetically homogeneous inoculum such as cloned DNA (83). The same is true for viruses that replicate via reverse transcription of an RNA intermediate, with the best-studied example being human and simian immunodeficiency viruses, which may generate variation of 10^{-2} per base upon propagation in a single individual (42, 167, 234).

Recombination is also common in both DNA and RNA viruses, including plant viruses (28, 39, 190, 313, 330). Among the latter, DNA recombination has been well documented in caulimoviruses and geminiviruses (28). Plant RNA viruses also recombine frequently, as judged from the analysis of sequence similarities in some genes of remote viruses (185), from studies of naturally occurring DI RNAs (190, 313, 362), and from experiments in which viable recombinants of nonviable parental genomes were selected (6, 39, 235, 270, 363). In RNA viruses, recombination by the copy choice mechanism relies on the intrinsic tendency of an RNA replication complex to dissociate and reassociate with the RNA template, resulting in occasional template switches (190, 313). Finally, plant pararet-

roviruses have a potential to undergo both host-mediated DNA recombination in the nucleus at the mini-chromosome stage and recombination by strand transfer during the course of reverse transcription. Indeed, experimental data compatible with both types of events are available (113, 345).

The low fidelity of RNA-dependent RNA and DNA synthesis is usually viewed as the result of the lack of proofreading activity in RNA replicases and in reverse transcriptases (324), as opposed to many DNA-dependent DNA polymerases (130). This might not be the fully adequate explanation, because reverse transcription can be performed in vitro by DNA polymerase I from *E. coli* with remarkable fidelity in the absence of proofreading (275), whereas the fidelity of different reverse transcriptases in vitro varies by 2 orders of magnitude (264, 265, 278).

It has been proposed that high rates of both misincorporations and aberrant strand transfers in animal retroviruses stem from the high ability of reverse transcriptase to add base-paired nucleotides to a 3′ nucleotide that is not base paired (255, 267, 330). One can speculate that this may also apply to RNA-dependent RNA replication.

The potential of plant RNA viruses and pararetroviruses to experience rapid genetic drift and occasional gross genome rearrangements was appreciated even before the advent of sufficient sequence data (350). Skepticism was expressed as to whether plant RNA virus vectors would amplify foreign genes faithfully. In a model experiment, mutants with deviant phenotypes could be easily recovered after a single virus passage, especially upon transfer to an unusual host (350). The evidence favoring the opposite view, i.e., that many virus mutants are remarkably stable in the absence of selection, was also pointed out (312). These two lines of argument were frequently alluded to (see, e.g., references 159 and 318). Often, nucleotide substitutions and DNA and RNA recombination are collectively referred to as genome instability. Analysis of more recent data may suggest that the impact of the two types of changes on the utility of plant virus replicons is quite different.

The enzymatic mechanism of RNA-dependent elongation of RNA and DNA chains is not sufficiently understood. The rate of nucleotide substitutions in these systems has been recently addressed (86, 90, 178, 234). In one comprehensive study, data from various experiments in vivo were collected and standardized (90). Importantly, data on retroviruses were taken from rigorous experiments with carefully designed vectors that replicated in the absence of selection (see, e.g., references 86 and 251). For a retrovirus life cycle that involves three error-prone acts of template synthesis and two strand transfers, mutation rates per base per replication were generally between 5×10^{-6} and 5×10^{-5} whereas the rate per genome per replication was from 0.04 to 0.4 (90). Values of the mutation rate upon RNA-RNA replication were substantially higher. However, the latter might be overestimations, because all experiments involved very short mutational targets (90).

To our knowledge, there is only one example in which the rate of spontaneous mutations of a long selectively neutral stretch of nucleotides in an RNA virus genome has been determined directly (178). For that purpose, a TMV vector that carried a copy of NPTII or a dihydrofolate reductase gene was used. In the absence of kanamycin or methotrexate, respectively, the added genes would not be under selection. The vector was inoculated onto tobacco plants, and cDNA copies of progeny genomes were subsequently cloned and sequenced. The interested reader should consult reference 178 for details of the calculation of the nucleotide substitution rate for TMV, which was estimated to be 4.5×10^{-6} to 1×10^{-4} per base per duplication. Substitution rates per genome per duplication (calculated as in reference 90) were approximately 0.03 to 0.6. These values are distinctly lower than estimated earlier (350). Clearly, they are quite close to the reliable values for retroviruses (86, 90, 251) and probably represent the accurate determinations of spontaneous mutation rates during RNA-RNA replication.

Although lower than thought, the mutation rates calculated above are substantially higher than for DNA genomes that are scanned by proofreading enzymes and, in addition, possess systems of nucleotide mismatch repair (89). From the population genetics point of view, such a mutation rate can be tolerated by a population only when recombination is allowed (89, 181, 182). Thus, in RNA viruses and in retroid viruses, low fidelity of replication enzymes necessitates a high rate of recombination, which is performed by the same enzymes.

When expressed per average gene (1,000 bp) per passage (~10 acts of duplication [see reference 171 for a discussion]), the substitution rate for the TMV vector will be below 10^{-1}; i.e., upon a passage in a plant, at most 10% of the inserts will experience a point mutation. Given that many of those will be silent, it may be estimated that a lot more than 90% of the copies of an insert will express a functional protein after that passage. All experimental evidence suggests that upon further passages but long before nucleotide substitutions would have inactivated a foreign gene, the insert will probably have been removed by recombination (51, 81, 84, 303).

Recombination rates in plant viruses, especially in the absence of selection, have not been subjected to extensive quantitative evaluation. On the other hand, a number of reports concern factors facilitating recombination. In homologous RNA-RNA recombination in bromoviruses, the ability of recombining strands to base pair appears to be essential (235–237). In contrast, recombination between turnip crinkle carmovirus and its satellites does not require RNA duplex formation but seems to be dependent on certain sequence motifs and elements of secondary structure (47).

Obviously, it would be advantageous to control recombination rates of RNA replicons. It has been noticed that DI RNAs, which result from RNA recombination by copy choice, are much more abundant and easily generated de novo in virus groups that do not encode a recognizable helicase homolog (e.g., carmoviruses and tombusviruses [185]) whereas they are less common in helicase-encoding viruses, although the viruses are able to replicate artificially constructed DI RNAs in *trans* (see, e.g., reference 269). This observation is compatible with the proposed role of helicases in maintaining accuracy of DNA and RNA synthesis (183). In one study, the rate of recombinational generation of DI RNAs de novo by the cloned genome of cucumber necrosis tombusvirus was strongly enhanced when expression of the viral nonessential 20-kDa protein was blocked (279).

Suppression of the accumulation of DI RNAs has also been reported. In broad bean mottle bromovirus, deletion derivatives of RNA 3 occur spontaneously. Passages of a strain containing these DI RNAs through a host in which the virus accumulates to low levels appear to eradicate the DI component (283). It is possible that reducing the virus replication rate while enhancing gene expression at another level (e.g., translation) will become a useful strategy for stabilizing gene insert expression. In another study, cymbidium ringspot tombusvirus and its genomic parasites were investigated (288). This virus supports replication of DI RNAs, derived from virus genomic RNA by copy choice recombination, and of satellite RNA that is unrelated to virus RNA. In plants in which the virus genome replicates together with its satellite RNA, formation of DI

RNAs was severely reduced, although replication of virus was not strongly impaired (288).

Undoubtedly, further examples of artificially altered recombination rates will be demonstrated. It will be interesting to find whether changes in the recombination rate in RNA viruses and pararetroviruses are followed by changes in the nucleotide substitution rate as a consequence of the same enzyme being active in both recombination and replication.

It can be concluded that the nucleotide substitution rate, whether alarmingly high, as had been suggested for RNA genomes, or comfortingly low in geminiviruses, is probably not the limiting factor in the effectiveness of plant viruses as expression vehicles. In contrast, recombination, whether by copy choice in RNA viruses, by template switch in pararetroviruses, or by a mechanism shared with the cellular genome, which is the case in pararetroviruses and geminiviruses, is a process which must be optimized further.

Biosafety Issues

Viruses are pathogens. Although some modified virus genomes, including many virus vectors, cause attenuated symptoms or no disease at all (41, 51, 82), release of any replicating entity into the environment must be monitored to ensure that it does not cause disease.

Recently, concerns about the biosafety of virus-derived sequences were expressed. A widely applied strategy of plant protection against viruses in field settings relies on the stable transformation of plants with a portion of the viral genome, most commonly with the virus coat protein gene. Expression of the transgene interferes with virus infection, probably by multiple mechanisms (reviewed in reference 368). It has been demonstrated that an incoming RNA virus can recombine with the RNA expressed by a transgene and acquire the coat protein gene from the plant mRNA (138). Capsid proteins often contain determinants for virus spread in plants and for natural transmission by insect or fungal vectors. Given that a number of plant lines transformed with viral capsid protein genes are already at the stage of field trials (368), scenarios in which recombinational events lead to viruses with new pathogenic features have been proposed (see, e.g., references 139 and 219).

More optimistic voices have countered by pointing out that any recombinational event that happens in transgenic plants is not mechanistically different from recombination between viruses in mixed infection in nontransgenic plants (38). It has been emphasized, however, that the means to quantify any harmful result of recombination are not available yet (219).

Hence, we await the results of additional experiments. Virus evolution is a fluid, ongoing experiment in recombination of a colossal magnitude which we can monitor on a very limited scale. It is notable, though, that traceable recombination events account for the emergence of several plant virus groups (185, 223). Release of transgenic crops strongly increases the frequency of one of the recombining alleles in the population. On the other hand, most plant cultivars, transgenic or not, could be used on average for a few dozen years, because resistance-breaking strains of pathogens appear as a result of genetic drift and selection (368). This limits the real time during which the undesired recombination between a virus and a transgene may be encouraged.

CONCLUDING REMARKS

As discussed in this review, small genomes of plant viruses encode a large repertoire of regulatory sequences that are useful for control of gene expression in plants. Because of their modular organization, many of these elements can be easily combined to create plant gene expression cassettes with novel specificities.

During the last few years, development of epichromosomal expression vectors based on replicating plant viruses has proved to be useful. Like any other vector, plant viruses have their own advantages and limitations. It seems safe to predict that plant virus vectors will be of considerable use in the laboratory, particularly when rapid expression and analysis of the fate of a protein in vivo are desirable and the pathogenic impact on the plant is less important.

At the same time, both virus genetic elements integrated into plant genomes and replicating viruses, when introduced into the environment, can increase the possibility of rapid evolutionary shifts in natural virus populations. Experiments that would allow estimates of such increases are desirable before any large-scale release of virus vectors and of modified plants is made.

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